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STUDIES ON THE THEORY OF DIABETES.

V. A STUDY OF NARCOTIC DRUGS IN PHLORHIZIN DIABETES.

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INTRODUCTION.

A fasting dog received 1 gram of phlorhizin suspended in olive oil subcutaneously once every 12 hours until the G: N ratio was constant, then once every 24 hours. This method, or the simple injection of 1 gram of phlorhizin once every 24 hours from the start, has been used extensively in this country for the preparation of animals for the study of gluconeogenesis and allied problems. By its use extensive data have been accumulated concerning the sugar-forming power of a wide range of substances. The above mentioned dog was etherized by E. A. Graham, who was studying the effects of anesthetics on the organs of animals whose glycogen deposits had been depleted. Without knowledge of what had occurred, one of us continued the urinalyses after the narcosis and found that the G: N ratio had risen from about 3.7 to 6.0. Our attention was then attracted by certain remarks which had appeared in an article by Dakin[1] concerning an animal similarly prepared. "A minimum amount of ether was given to the dog as an anesthetic during the exposure of the vein. Under these conditions a marked increase in glucose followed the administration of histidine, but later experience would indicate that at least some of this glucose did not originate from the base but was due to the anesthetic and to metabolic disturbances following the intravenous injection."

We then made experiments to learn the constancy of such occurrences and found that following ether narcosis there was regu-

[1] H. D. Dakin: this *Journal*, xiv, p. 328, 1913.

larly a striking increase of the glycosuria, and that nitrous oxide had similar effects. Moreover, the records of the experiments were such that, had they been obtained after the subcutaneous injection of some supposed intermediate, such, for example, as a pyruvic acid salt, they would doubtless have passed as evidence that the substance itself had been transformed into sugar (compare Experiment IV). For nitrous oxide this was impossible, and for ether highly improbable.

The question then arose as to how many other substances might react like ether or nitrous oxide, and how to avoid confusing effects of this type with true transformation of the administered substance into sugar. The following study was made for the purpose of answering these questions. Incidentally it was recognized that phlorhizinized dogs might offer certain advantages for the study of the metabolic changes which occur under the influence of narcotic drugs generally. The problem has led us to investigate a method of preparing phlorhizinized dogs, which differs from that above described by the supplemental injection of epinephrin every 3 hours until this drug has no further power to affect the glycosuria. It is believed that certain of the substances which have come to be recognized as sugar formers on the basis of data obtained by the other method must be retested by the amplified method or its equivalent.

The source of the extra sugar.

It has been observed by Lusk[2] that fasting and phlorhizin alone are incapable of removing all glycogen from the body. This is confirmed by the results of the present study. Accordingly, the phlorhizinized animals which showed an increase of the glycosuria after narcosis still contained glycogen, and it was first necessary to investigate this as a possible source of the extra sugar.

Ringer, working with Lusk, gave epinephrin intraperitoneally to dogs which had been fully phlorhizinized and made to shiver until further treatment caused no further output of extra sugar. Epinephrin then caused no increase of the glycosuria. Another animal was treated less vigorously with cold and then given epinephrin. In this case extra sugar was excreted, but with successive doses of epinephrin the increases in glycosuria

[2] G. Lusk: *Ergebn. d. Physiol.*, xii, p. 361, 1912.

became less and finally approached zero. From this it was inferred that epinephrin swept out residues of glycogen, if such were present, but that in deglycogenized animals it had no power to increase the glycosuria or otherwise affect carbohydrate metabolism. Undertaken originally to test the validity of Eppinger's[3] claim that epinephrin affects the pancreas and promotes a new formation of sugar from fat, which was not supported, these studies incidentally showed that a dog may be deglycogenized by epinephrin in the same sense that this is possible by cold or muscular work. Lusk[4] has since shown by respiratory calorimetric experiments the correctness of this interpretation.

The method of deglycogenizing dogs by the combined effects of phlorhizin and cold baths,[5] followed by prolonged shivering, is effective but arduous. We have used it in previous studies[6] and confirmed by post-mortem tissue analyses the view that the muscles and liver may be thus freed of all measurable traces of glycogen. But the regimen weakens the dogs and subsequent experiments frequently terminate prematurely, particularly when the animal is not powerful, or if the experimental substance administered is toxic. Furthermore, it is never possible to state certainly that the cold has been pushed to the point of complete deglycogenation except by administering it again during a control period and finding no output of extra sugar. To do this means usually to risk a possible loss of urine.

Experiments were now made to learn whether epinephrin might be used conveniently in place of cold, with the idea that its administration would not entail removing the dog from the cage and that it might be possible to control the amount given with greater ease.

In the first experiment an animal was given 1 gm. of phlorhizin every 24 hours as usual, supplemented by a cold bath for 20 minutes, followed by moderate shivering for 3 hours. The first day's urine was discarded, but the G: N ratios found in subsequent 12 hour periods were 5.72, 3.90, 3.82, and 3.67 (Experiment VII). Epinephrin was now administered, 1 mg. subcutaneously, once every 6 hours, *i.e.*, 1 cc. of 1 to 1000 adrenalin solution (Parke, Davis), and during the following 12 hour periods the ratios were 8.82, 4.32, 4.14, and 3.73 respectively. Under this system the ratio was

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[3] H. Eppinger, W. Falta, and C. Rudinger: *Ztschr. f. klin. Med.*, lxvi, p. 1, 1908.

[4] Lusk: *Arch. Int. Med.*, xiii, pp. 673–681, 1914.

[5] Lusk: *Ztschr. f. physiol. Chem.*, lxvi, p. 106, 1910.

[6] R. T. Woodyatt: this *Journal*, xiv, p. 441, 1913.

still not certainly constant after 48 hours of epinephrin injections. The dog was nevertheless used at this point for an experiment. Nitrous oxide narcosis was induced and maintained for 2 hours. "Extra sugar" for the period was 1.94 gm. In previous experiments the same dog prepared with phlorhizin alone had excreted 8.82 and 7.24 gm. of "extra sugar" after two nitrous oxide narcoses of the same duration (Experiments V and VI).

In a second instance a dog was phlorhizinized as usual with no supplementary treatment. In the sixth 12 hour period G: N reached 3.70, in the seventh, 3.65. The dog now received 1 mg. of epinephrin subcutaneously once every 2 hours, and the ratios by 6 hour periods ran 10.75, 4.52, 3.09, and 3.04 (Experiment XII). Thus after 12 hours of the epinephrinization G: N was again constant and *at a level below that of the fore-periods.*[7]

Later it was found less injurious to give epinephrin once every 3 hours from the beginning of the phlorhizinization. A dog received phlorhizin as usual, and also 1 mg. of epinephrin subcutaneously every 3 hours. The first day's urine was discarded. That for the two following 12 hour periods showed ratios of 2.93 and 2.96 respectively. In the next period epinephrin was omitted and the ratio was 2.87. It was then resumed as before, and the ratio was 2.90. After this the same method was adhered to. As a rule, when 1 cc. of adrenalin solution was given every 3 hours from the beginning of the preparation, G:N reached constancy in 18 to 24 hours, after which epinephrin could be omitted and resumed without changing the ratio.[8]

A dog which had been so prepared was anesthetized for 30 minutes with ether and showed no increase of the glycosuria, nor any material rise in

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[7] This would seem to suggest the possibility that in this experiment, during the periods of higher ratios, a part of the glucose in the urine was attributable to a slight but steady mobilization of sugar from glycogen. If not, it would have to be assumed that the epinephrin or the tissue asphyxia produced by it altered the protein metabolism or the excretion of its end-products.

[8] In later experiments the same method resulted in the premature death of several dogs. The dose of epinephrin was accordingly reduced to 0.25 mg. at the same intervals, after which results were again obtained like the earlier ones. The fatalities began with the introduction into the laboratory of a new supply of adrenalin. Doubtless the solutions used before had lost strength. By using the material supplied in small ampules, a fresh one for each dose, we have had uniformly satisfactory results with 0.25 mg. of epinephrin every 3 hours, up to the time of the present writing. It has also been found that the epinephrin need not be given continuously every 3 hours, but that it may be given during the day, omitted at night, and finished the next day, without requiring in all more doses of the drug. This harmonizes with Lusk's observation that there is no storage of glycogen during complete phlorhizinization.

the G: N ratio (Experiment III); whereas animals prepared by simple phlorhizinization and anesthetized for 10 minutes, 30 minutes, and 2 hours, showed outputs of 3.88, 7.98, and 11.26 gm. of extra sugar respectively (Experiments I, II, IV).

From these experiments it was concluded that the extra sugar which is secreted after ether or nitrous oxide narcosis in phlorhizinized dogs has its origin in glycogen.

Mechanism of the increased glycosuria.

It is common knowledge that glycosurias may follow prolonged exposure of healthy animals or men to the effects of ether, chloroform, chloral, paraldehyde, acetone, urethane, morphine, or other narcotic drugs. Such glycosurias are attended by *hyperglycemia.* So far as studied the hyperglycemias have been found to depend on a discharge of *glucose from glycogen,* but this has been sharply proved only for a few narcotics. As a rule, these hyperglycemias and glycosurias reach a marked height only with general narcosis attended by signs of *asphyxia.*

On the basis of a long series of experiments Oppermann[9] concluded that narcotics in general, when in small concentration, lower the blood sugar concentration, but when in the high concentrations productive of deep general narcosis they cause hyperglycemia, which, if sufficiently great, leads to glycosuria. Pawel[10] has recently shown that paraldehyde in merely soporific doses slightly depresses the blood sugar concentration, but in heavy doses with general narcosis it elevates it sometimes sufficiently to cause glycosuria. Seelig,[11] who studied ether glycosuria, found that by administering oxygenated salt solution intravenously during the anesthesia the tendency to glycosuria was decreased, and he attributed the glycosuria of ether narcosis to asphyxia. Müller,[12] working with acetone, found the more marked glycosurias only when the animal became cold and asphyxiated. Oppermann[13] also expresses the view that narcotic drugs produce glycosuria only during deep narcosis with asphyxia. Naunyn classifies the narcotic glycosurias among the asphyxial glycosurias. Ruschhaupt[14]

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[9] F. Oppermann: *Deutsch. Ztschr. f. Nervenheilk.*, xlvii–xlviii, p. 590, 1913.

[10] E. Pawel: *Biochem. Ztschr.*, lx, pp. 352–369, 1914.

[11] A. Seelig: *Arch. f. exper. Path. u. Pharmakol.*, lii, p. 481, 1905; liv, p. 206, 1906.

[12] Cited from B. Naunyn: *Der Diabetes melitus*, Vienna, 2d edition, 1906, p. 52.

[13] Oppermann: *loc. cit.*

[14] Cited from Naunyn: *loc. cit.*

found that acetone narcosis failed to produce glycosuria in dogs whose glycogen reserve had been depleted by fasting. King, Chaffee, Anderson, and Redelings[15] have shown that the sugar which is excreted in ether glycosuria comes from glycogen. *These and other contributions to the literature indicate that, when given to healthy animals in sufficient doses, narcotic drugs induce an hyperglycemia with or without glycosuria, that the rise in blood sugar is at the expense of glycogen, and that they produce their effect by causing asphyxia.*

There is no reason to believe that narcotic drugs do not produce asphyxia in phlorhizinized dogs as well as in others, or that such asphyxia is less able to mobilize glycogen if the latter is present. The effect of this in a phlorhizinized dog would be a tendency toward hyperglycemia as in non-diabetic animals, but in this case a rapid transfer of all extra sugar to the urine. *We would therefore regard the increase of glycosuria following narcosis in phlorhizinized dogs as identical in mechanism with narcotic glycosurias generally and hence as due probably to asphyxia, with whatever acid increase this implies.*

It is concluded from the foregoing that any substance or procedure capable of creating hyperglycemia at the expense of glycogen in a non-diabetic animal might increase the glycosuria of a totally diabetic but imperfectly deglycogenized dog in the same way. Naturally, free acids, narcotic substances, and procedures or substances generally which can induce tissue asphyxia, might lead to the appearance of extra sugar in dogs prepared as described on page 1, without affording any evidence of *newly formed* sugar. Among the aldehydes, ketones, keto-acids, and alcohols, which have come to be regarded as sugar formers, many are narcotic. The basis for considering them gluconeogenetic has been in several instances their observed effects on the urine of phlorhizinized dogs *whose freedom from glycogen was not established.* In some instances the methods used were incapable of removing glycogen. We suggest that when studying the sugar forming power of substances in phlorhizinized dogs the presence or absence of glycogen should be ascertained by giving 0.25 to 1 mg. of epinephrin once or twice subcutaneously during one of the fore-periods. If no extra sugar appears in this period, it is safe to assume that a

[15] J. H. King, B. S. Chaffee, D. B. Anderson, and L. H. Redelings: *Bull. Johns Hopkins Hosp.*, xxii, p. 388, 1911.

rise in glycosuria following the administration of any other substance is not referable to glycogen. In human diabetics and depancreatized dogs the presence of glycogen might be similarly detected.

Suppressions.

Besides an increase of glycosuria following narcosis, *suppressions of nitrogen, glucose, and the acetone bodies* were sometimes observed. They were not constant, but varied with the drug used and with the conditions of the experiment.

1. *Suppressions of glucose and nitrogen.* Fully phlorhizinized and epinephrinized dogs, when anesthetized with ether, showed a lessened excretion of glucose and nitrogen beginning with the ether period and lasting for 24 hours or more (Experiment III). The same was true in two of the dogs which had received phlorhizin but no epinephrin, with one difference, namely, that the fall in glucose first appeared in the after-periods; because in the ether period itself any possible tendency toward decreased glycosuria was masked by the increased supply of glucose derived from glycogen (Experiments I and IV). When the glycogen had been discharged by epinephrinization the fall in glucose following ether narcosis parallelled that in nitrogen and the G: N ratio remained unchanged (Experiment III). From this it is evident that *the suppressions of nitrogen and glucose are not inseparable from the excretion of extra sugar.*[16]

This fact is emphasized by the observation that with nitrous oxide there were no secondary suppressions of glucose and only slight suppressions of nitrogen, even when the initial increase in glycosuria was very pronounced (Experiments V, VI, VII). In one of the ether experiments also with a marked increase of glycosuria, the fall in nitrogen was comparatively small and the primary increase in glucose was followed by a return of the glucose to the same level as in the fore-periods (Experiment IV), not by a fall below the level of the fore-periods as in Experiments I, II,

[16] Working with other narcotics (aldehydes) Ringer and Frankel regard the increase of glucose and the suppressions (of acetone bodies) as inseparable effects of a single specific chemical reaction between aldehydes and β-hydroxy butyric acid. Our paragraphs on "*suppressions of the acetone bodies*" and "*acetaldehyde*" bear further on this view.

III. These decreases in glucose and nitrogen might be ascribed (a) *to a general slowing of the whole protein metabolism;* (b) *to retentions.* The latter is probably the chief process concerned.

(a) *General slowing of the protein metabolism.* During sleep the metabolism as a whole is somewhat decreased (Benedict[17]) and in drug narcosis the rate of tissue respiration is lessened, but, nevertheless, heavy doses of narcotics generally cause an increased output of nitrogen in the urine. Mild or merely soporific doses do not exhibit such effects, and it is commonly said that the increased protein breakdown, is a result of deep toxic effects of the narcotic drug comparable to those of arsenic, phosphorus, and other members of the so called "oxidation limiting" group.[18] These deep effects, like the glycogen mobilizations, are intimately related to the degree of asphyxia and acid produced. Unless these substances react differently in the diabetic than in the normal animal, there would be no basis for assuming that they may *limit* the protein catabolism as a whole. Ray, McDermott, and Lusk[19] found that there was indeed some difference between the effects of phosphorus in non-diabetic and diabetic animals, in that phosphorus did not further increase the protein breakdown when this had already been driven to a maximum by phlorhizin. But even this does not imply any effect which could be called a general inhibition.

(b) *Retentions.* Since the term retention is broad and one which is differently understood by different writers, it is necessary to make clear the sense in which it is here used.

Local or tissue retention. "Retention" is employed in this paper to denote, not merely the damming back of urinary substances in the blood and other tissues by failure of kidney excretion, but also the holding back of such substances by the tissues themselves. Such retention is not necessarily accompanied by an increased concentration of urinary substances in the blood. It may, however,

[17] Benedict: *Carnegie Institution of Washington, Publication No. 203,* 1914. (In press.)

[18] For the literature on which these remarks are based the reader is referred to Von Noorden's *Handb. der Path. d. Stoffwechsels,* Berlin, 2d edition, 1906, ii, Chap. xv.

[19] W. E. Ray, T. S. McDermott, and G. Lusk: *Am. Jour. Physiol.,* iii, p. 139, 1899–1900.

accompany the swelling of tissues with absorption of water, salts, etc., which constitutes edema, the latter term being used in the sense suggested by M. H. Fischer to denote a change in the character of the tissue colloids due to local interactions between them and certain types of chemical substances important among which are acids.

Chloroform is prone to cause swelling of cells, with fat infiltration, necrosis, a hemorrhagic tendency, etc. Ether has not been observed to cause necrosis, but it may produce milder forms of parenchymatous degeneration and tissue swelling. Nitrous oxide has little tendency to produce any visible tissue changes. The tendency of chloroform, ether, and nitrous oxide to produce suppressions of glucose and nitrogen excretion in phlorhizinized dogs parallels their ability to produce changes in which tissue swelling is an important feature. Accordingly it might appear reasonable to suggest that these suppressions represent *retentions* by swollen tissues.[20]

The visible changes in the tissues during narcotic poisoning are accompanied by fairly characteristic metabolic changes. Thus, chloroform and phosphorus given to non-diabetic animals not only increase urinary nitrogen as a whole, but alter the nitrogen partition. The same effect on the urine accompanies acute yellow atrophy of the liver, and is seen less often after prolonged ether narcosis and heavy doses of other narcotic drugs. While there are doubtless differences between the urinary changes

[20] Working with chloroform it is difficult to narcotize phlorhizinized dogs for more than 8 to 10 minutes without killing them; although with ether, narcosis was successfully prolonged for 2 hours. Even with the shorter chloroform narcoses, all urinary secretions promptly fell to so low a level as to mask any urinary evidences of a possible glycogen mobilization. The great susceptibility of phlorhizinized dogs to chloroform is referred to the absence of the protective influence of glycogen. Graham, in this laboratory, produced characteristic pictures of chloroform poisoning in the kidneys and liver of phlorhizinized dogs with quantities of chloroform too small to exert such effects in well nourished animals. These experiments, in conjunction with the above mentioned suppressions, incidentally point to the danger which attends the use of chloroform in poorly nourished individuals. Ether is only less likely to produce marked suppressions and visible changes in the organs. Nitrous oxide has proved relatively incapable of causing such changes. In a necessary operation upon a diabetic individual it would seem that nitrous oxide should have preference.

encountered, on the one hand, after poisonings with different members of the group of drugs under discussion, and, on the other, in acute yellow atrophy and similar liver diseases, they have certain points in common, so that in general it may be said that *the percentage of urea nitrogen is decreased and the percentage of "rest" nitrogen increased.*

Salkowski[21] found in the urine of a case of acute yellow atrophy a large quantity of nitrogen in colloid but non-coagulable form. Mancini[22] also found an increase of the colloid nitrogen of the urine in different liver diseases. In two cases of phosphorus poisoning Sjôquist found urea 55.1 and 60.6 per cent; NH_3 27.6 and 14.2 per cent; "rest" N 17.3 and 26.2 per cent.[23] Howland and Richards[24] found in chloroform poisoning that the undetermined nitrogen of the urine was markedly increased, while urea showed a relative decrease or even an absolute decrease in spite of the great rise in total nitrogen, and observations tending similarly have been made for a number of other narcotic drugs.

From the above considerations it is clear that following the administration of drugs like chloroform and ether to diabetic dogs, there occurs a series of changes in the tissues, notably the liver, kidneys, and blood, which involves alterations in their physicochemical and purely chemical composition, also even in their gross physical characteristics. All these changes are intimately connected. They closely resemble the changes produced by asphyxia and acid, and merge insensibly with the problem of edema. It is concluded that such changes afford ample material with which to construct explanations for the observed suppressions of glucose and nitrogen, but that details of the mechanism can scarcely be discussed further than to suggest that *the suppressions in question represent retentions due to the effects on the tissues of an asphyxia- and edema-producing drug.*

2. *Suppressions of the acetone bodies.* If the suppressions of glucose and nitrogen are correctly interpreted as retentions in the sense indicated, this implies the existence of conditions which

[21] E. Salkowski: *Berl. klin. Wchnschr.*, xlii, p. 1581, 1905.

[22] S. Mancini: *Arch. di farm. sper.*, v, pp. 395–407, 1906.

[23] Cited from Wiechowski in Neubauer-Huppert's *Analyse des Harns*, Wiesbaden, 11th edition, 1910, p. 498.

[24] J. Howland and A. N. Richards: *Jour. Exper. Med.*, xi, pp. 344–372, 1909.

would necessarily diminish the output of acetone bodies. That this actually occurred can occasion no special comment or be regarded as evidence of any additional process, unless, since the acetone bodies arise not wholly from protein, but in part from fats, it might be suggested that the narcotics derange the fat metabolism along with that of protein and as part of the same general intoxication.

Acetaldehyde.

Ringer and Frankel[25] gave acetic and propionic aldehydes to dogs which had been phlorhizinized in the way outlined on page 1. They noted an increase of glycosuria in the aldehyde periods, and a decrease in nitrogen lasting 12 to 24 hours after the aldehyde administration, accompanied by a fall in the acetone body output. Reference to their protocols will also show that following the increase in glucose there was at times a secondary fall to a point below the level of the fore-periods, similar to those described by us for ether, although these writers do not discuss this feature.

In their Experiment XXIX (p. 578), glucose for the last fore-period is 17.59 gm.; for the 12 hours in which acetaldehyde was given, 24.85 gm.; for the next 12 hours, 13.90 gm. Similarly in Experiment XXVII (p. 575); Experiment XXVIII, periods 14 and 15 (p. 576); and in Experiment XXX (p. 578).

Ringer and Frankel propose to interpret these results as follows: "Extra sugar," reckoned as the difference between the number of grams of glucose excreted in the experimental period and the number of grams of nitrogen times G: N (when G: N equals the average G: N ratio found in the control periods), represents literally so many grams of newly formed sugar, regardless of whether this value has its counterpart in a corresponding absolute increase of the glycosuria or not. "Extra sugar" so reckoned having in some instances exceeded the 12 grams which might theoretically be derived from the 8.8 grams of acetaldehyde given, acetaldehyde therefore promotes a new formation of sugar from fat. It does this, they suggest, by entering into a glucoside-like union with β-hydroxy butyric acid at the aldehyde group, to yield a new sub-

[25] A. I. Ringer and E. M. Frankel: this *Journal*, xvi, pp. 563–579, 1913–14.

stance which is gluconeogenetic. Incidentally this substance is non-ketogenetic. Hence the increase of glycosuria and the fall in the acetone bodies are both expressions of one and the same chemical mechanism, a reaction for the fulfillment of which substances containing aldehyde groups are essential. In this paper and in a later one Ringer[26] assigns to hypothetical glucoside unions of this type a deep biologic significance and proposes that sugars and all so called antiketogenetic substances contain aldehyde groups and operate in this way; also that failure to consummate such glucoside-like unions lies at the bottom of all chemical disturbances in diabetes.

There are many obvious objections to this hypothesis of antiketogenesis and diabetes, but we shall here discuss somewhat in detail only the experimental work upon which it is based. In the first place, a comparison of the protocols of these aldehyde experiments with those of the present series with ether in which the technique of phlorhizinization was identical (Experiments I, II, III) will show that ether may produce effects which differ in no essential from those obtained by Ringer and Frankel. Accordingly it is clear that such changes are in no sense specific for substances containing aldehyde groups. Secondly, acetic and propionic aldehydes are narcotic substances. Acetaldehyde, with its close relationship to paraldehyde and chloral, is particularly easy to identify as such, and, in harmony with data to be found in textbooks, we find that guinea pigs may be completely anesthetized by acetaldehyde inhalation much as with ether, although the stage of excitement is more violent. It would seem reasonable to expect, therefore, that the changes produced in the urine of phlorhizinized dogs by this substance should be interpreted along such lines as have already been suggested for narcotics generally. If so, acetaldehyde should fail to increase the glycosuria in phlorhizinized and completely deglycogenized dogs.

Experiments were made by us with acetaldehyde in phlorhizinized dogs that had been epinephrinized as described on page 4 (see Experiments VIII, IX, X, XI).[27] In Experiment VIII, and in all others, 8.8 grams of acetaldehyde were given subcuta-

[26] A. I. Ringer: *ibid.*, xvii, pp. 107–119, 1914.

[27] A considerable part of this work was carried out by Dr. Erich Schwartze, whose assistance is herewith acknowledged with pleasure.

neously as in the experiments of Ringer and Frankel. During the acetaldehyde period there was a very slight total increase in glucose with no significant change in nitrogen, hence a slight rise in G: N and "extra sugar" not to exceed 0.42 gram. The acetone bodies remained constant. In the 12 hours *following* the aldehyde period the glucose returned to its original level, but the nitrogen and acetone bodies fell somewhat and the G: N ratio rose correspondingly, while "extra sugar" figured literally as the grams of glucose minus the product (grams nitrogen by the average G: N ratio of the fore-periods) would be for this period 1.3 grams. In Experiment IX there was, following the aldehyde administration, a decrease in the total glucose from 26.10 to 22.98 grams, nitrogen falling in proportion and G: N remaining constant at 3.20 and 3.17. In the next 12 hours the fall in glucose continued, but now the nitrogen fell even more markedly as in Experiment VIII, thus increasing the G: N ratio from 3.17 to 5.30. After this period the dog was moribund. Although there was a total decrease in glucose, if "extra sugar" is figured in the way described, it would appear as though 8.36 grams of new sugar had been formed. In Experiment X there were similar decreases of glucose and nitrogen, with a rising G: N ratio and "extra sugar" at 4.10 grams, in spite of an absolutely decreased output of glucose. Here again the experiment was terminated by the animal's condition. In Experiment XI the glucose was reduced from 12.90 to 6.90 grams and nitrogen from 4.31 to 1.15 grams, so that the ratio rose from 3.0 to 6.0. Again the dog died.

In Ringer and Frankel's Experiment XXX (p. 578), there was, as in the present series, no significant total increase of the glucose, but the usual fall in nitrogen, a rising ratio and "extra sugar" reckoned at 3.2 grams. Since no records of an after-period are recorded, the experiment perhaps was ended in this case also by the animal's condition.

In none of these experiments was there an actual significant increase of the glycosuria. Even "extra sugar," literally interpreted, never exceeded the 12 grams which could be formed if all the carbon in the acetaldehyde had been converted into glucose. That this might occur in incompletely deglycogenized dogs is, however, evident from experiments with nitrous oxide and epinephrin. Here *all* the carbon in the substances given was nil or neg-

ligible. It is a curious fact that Rudinger, Eppinger, and Falta, for this reason, held that epinephrin was capable of causing a new formation of sugar from fat, a fallacy which Ringer himself was instrumental in demonstrating.

The question arises as to whether an increased G:N ratio can be regarded as evidence of any new formation of sugar at all, when it occurs, as in these experiments, with a stationary or falling glucose output, and sometimes delayed. We think not. As shown on page 10, the known effects of narcotic drugs on the protein metabolism and the viscera are sufficient to account for such changes. Similar phenomena may be encountered in the late stages of experiments in which only phlorhizin and epinephrin are given, an example of which is presented in periods 18 and 19 of Experiment XII. Ritzmann[28] has shown that the power of epinephrin to mobilize glycogen probably rests on the asphyxia which results from the constriction of vessels; *i.e.*, presumably on an increased acid content in the glycogen-containing organs, notably the liver. As already pointed out, narcotic hyperglycemias may be referred likewise to asphyxia (and hence acid). Is it remarkable that with prolonged epinephrinization further changes should appear analogous to those seen with deep narcotic intoxication?

It is concluded that the experiments offer no evidence that acetaldehyde is itself convertible into glucose or capable of promoting any new formation of sugar, which is in harmony with the observations of Friedmann[29] that in liver perfusion experiments acetaldehyde is an acetone former, and not a sugar former. It is also concluded that the hypothesis that acetaldehyde promotes a new formation of sugar from fat has no more support than that which assigns to epinephrin a similar power. The hypotheses of antiketogenesis and diabetes which are based on the assumption that acetaldehyde promotes a new formation of sugar from fat are wholly untenable.

[28] H. Ritzmann: *Arch. f. exper. Path. u. Pharmakol.*, lxi, p. 231, 1909.
[29] Friedmann: *Biochem. Ztschr.*, xi, p. 145, 1913.

SUMMARY.

Dogs were prepared (A) by fasting and 12 to 24 hourly subcutaneous injections of 1 gram of phlorhizin suspended in olive oil until the G: N ratio in the urine became constant, and (B) by the same method plus 2 or 3 hourly subcutaneous doses of epinephrin until these no longer affected the glycosuria or the G: N ratio.

The experiments confirm earlier observations of Lusk and his pupils that fasting and phlorhizin alone do not remove all glycogen from the body, but that this may be done by supplementary doses of epinephrin.

In dogs prepared by (A) narcosis with ether or with nitrous oxide was followed by excretion of extra sugar, but not in dogs prepared by (B). The extra sugar, therefore, comes from glycogen. The effect is equivalent to the narcotic hyperglycemias and glycosurias of non-diabetic animals, and probably depends on the degree of tissue (liver, muscle) asphyxia produced, with whatever acidity this implies; *vice versa*, ether and nitrous oxide hyperglycemias are due wholly to mobilizations of glycogen.

Method A, as used by a number of writers, is not reliable for the study of *new sugar* formation following administrations of substances that may cause hyperglycemias in non-diabetic animals; (notably free acids, certain alcohols, etc.), or substances that favor tissue asphyxia generally, since they might lead to the appearance of extra sugar from glycogen.

Following ether narcosis there were lessened excretions of nitrogen and the acetone bodies, and, following the preliminary increases of glucose when such occurred, lessened outputs of glucose also. Suppressions were minimal after nitrous oxide, maximal after chloroform; they parallelled the toxicity of the drug used and represent retentions due to anomalies of metabolism and excretion associated with tissue injuries and edemas, similar to those produced by asphyxia and acid.

Following ether narcosis in dogs prepared by A, the results were similar to those obtained and differently interpreted by Ringer and Frankel following aldehyde administration to dogs, also prepared by A. Experiments with acetaldehyde by method B have given no support to the hypothesis that this drug leads to *new formations* of sugar from fat or any other source, or that its

effects are peculiar to substances containing aldehyde groups, as suggested by these writers. The hypothesis that antiketogenesis and diabetic phenomena generally depend upon the occurrence or non-occurrence of glucoside-like unions between aldehydes and alcohol groups is also wholly untenable.

If 0.25 to 1 mg. of epinephrin is given subcutaneously during the fore-period of experiments with diabetic dogs, it serves to reveal the presence or absence of glycogen. This is suggested as a procedure which might well be used as a check in all experiments on diabetic animals in which the mobilization of glycogen could lead to erroneous conclusions.

Method B has certain practical advantages over the method of combining the effects of phlorhizin and cold, in that the animal need not be taken from the cage, and the risk of losing urine is eliminated. The objection that night work is necessitated by epinephrin injections every 3 hours for 18 to 24 hours may be removed by giving the epinephrin for an equivalent time on successive days.

EXPERIMENTS.

In all the experiments the animals were bull terriers weighing 15 to 25 kg. The epinephrin was Parke, Davis & Co.'s adrenalin 1:1000 solution with chloretone, of which 1 cc. was taken as 1 mg.; phlorhizin (Merck's); acetaldehyde (Kahlbaum's); chloroform (Malinckrodt's "anesthetic"); ether (Squibb's); nitrous oxide (that prepared for anesthetic purposes by the Chicago Oxygen Gas Co.).

The animals were kept without food and the urine was removed from the bladder by catheter, with subsequent irrigation, at such intervals as were found necessary to preclude urination in the cage, and for sharp separation of periods.

Analytical methods. Glucose was determined by titration according to Bang, and by polariscope in every instance. After drug administrations these methods were carried out before and after fermentation with yeast and supplemented by readings in Lohnstein saccharimeters. Since the inclusion of the figures obtained by these methods neither adds to nor detracts from the significance of the titration figures, only the latter are given in the tables. Nitrogen was determined by Kjeldahl, acetone and aceto-

acetic acid by the conventional distillation iodine-thiosulphate method. β-Hydroxy butyric acid was determined by ether extraction, according to Black, the residue obtained after separation of the ether being then subjected to Shaffer's bichromate oxidation method.

"Extra sugar" on the charts refers always to the difference G − (N × G: N), in which G = grams of glucose for the period; N = grams of nitrogen for the same period; and G: N = the average G: N ratio for the two periods preceding, unless otherwise noted.

"Gross sugar increase" is the difference between the grams of glucose for a given period and the average glucose excretion in grams for the two preceding periods.

Experiment I. Ether. The dog received 1 gm. of phlorhizin suspended in olive oil subcutaneously once every 24 hours. When two successive periods had shown ratios of 3.97 and 3.76 respectively, the dog was kept asleep by ether inhalation during four separate 30 minute intervals. Note (1) the primary increase in glycosuria in the ether period with subsequent suppression in period 4; (2) depression of nitrogen in periods 3 and 4, with rising tendency in later periods; (3) depression of acetone bodies in ether period with later steady rise. These results are essentially those seen by Ringer and Frankel with acetaldehyde.

12 HR. PERIOD	GLUCOSE	NITROGEN	G:N	"EXTRA SUGAR"	GROSS SUGAR INCREASE	ACETONE†	β-HYDROXY BUTYRIC ACID	TOTAL* ACETONE BODIES	REMARKS
	gm.	gm.		gm.	gm.	gm.	gm.		
1	19.25	4.84	3.97			0.123	0.117	0.338	
2	16.75	4.45	3.76			0.134	0.113	0.354	
3	*25.62*	*3.72*	*6.88*	*11.26*	*7.62*	*0.086*	*0.066*	*0.220*	Ether narcosis 2 hrs.
4	12.44	3.39	3.67			0.229	0.042	0.453	
5	10.75	3.56	3.02			0.229	0.110	0.521	
6	12.00	3.92	3.03						
7	10.88	3.47	3.13						
8	13.13	4.12	3.19						

† Inclusive of aceto-acetic acid.
* Reckoned in terms of β-hydroxy butyric acid.

Experiments II, III, and IV. Ether. The ether was administered in each case by inhalation. In Experiments II and IV the preparation was the same as in Experiment I. But in Experiment III (in the center) the dog was given epinephrin until it could be omitted and resumed without affecting the G: N ratio before narcotizing.

EXP. NO.	12 HR. PERIOD	GLUCOSE	NITROGEN	G:N	"EXTRA SUGAR"	GROSS INCREASE IN GLUCOSE	DURATION OF NARCOSIS	REMARKS
		gm.	gm.		gm.	gm.	min.	
II	1	17.95	4.71	3.81				Note steady fall in N with primary rise followed by fall in glucose, also changed ratio in period 5.
	2	12.29	3.88	3.16				
	3	12.63	4.15	3.04				
	4	*15.29*	*3.68*	*4.15*	*3.88*	*2.83*	*10*	
	5	6.78	2.29	2.52				
III*	1	23.40	8.37	2.79				Epinephrin 1 mg. every 2 hrs.
	2	23.38	8.79	2.65				Epinephrin omitted.
	3	*21.13*	*7.78*	*2.71*	*0.0*	*0.0*	*30*	*Epinephrin as above. Ether narcosis.*
	4	17.40	6.01	2.89				Epinephrin omitted.
	5	13.85	4.96	2.74				" "
IV	1	20.25	7.25	2.79				
	2	21.62	7.36	2.93				Note primary rise in glucose, with subsequent return to level of fore-period. Also the moderate fall in nitrogen.
	3	*27.63*	*6.87*	*4.06*	*7.98*	*6.69*	*30*	
	4	21.62	6.89	3.14				
	5	22.00	6.64	3.31				
	6	19.81	6.82	2.90				
	7	18.18	5.29	3.43				
	8	17.81	5.32	3.35				
	9	16.75	5.49	3.05				
	10	16.62	5.10	3.25				

* Note on preparation of animal for Experiment III. The dog received phlorhizin 1 gm. suspended in olive oil, subcutaneously once every 24 hours until the G:N ratio became constant. It then received epinephrin 1 mg. subcutaneously every 2 hours. At first the epinephrin caused a heavy output of extra sugar, but ultimately (after 18 hours) G:N was again constant. The protocol shows the record from this time on.

Experiments V and VI. Nitrous oxide. The dog was phlorhizinized as in Experiment I. In the second half of the third 12 hour period of virtually constant ratios the dog was kept asleep for 2 hours by means of N_2O inhalations. The chart shows 12 hour periods and also analyses for the 6 hour half periods including the narcosis. In the first half of period 5 the narcosis was repeated as before. Note the absence of any marked decrease in nitrogen or marked fall in sugar following initial rise, also steady increase of the acetone bodies.

12 HR. PERIOD	GLUCOSE		NITROGEN		G:N	"EXTRA SUGAR"	ACETONE		β-HYDROXY BUTYRIC ACID	TOTAL ACETONE BODIES	REMARKS
	gm.		*gm.*			*gm.*	*gm.*		*gm.*		
1	12.25		4.40		2.78		0.060		0.164	0.272	
2	13.94		4.73		2.94		0.079		0.155	0.297	
3	*22.06*	7.81 *14.25*	*4.73*	2.35 *2.38*	*4.87*	*8.82*	*0.091*	0.041 *0.050*	*0.202*	*0.366*	2 hrs. narcosis.
4	12.88	6.69 6.10	4.31	2.06 2.25	2.99		0.162	0.064 0.098	0.299	0.590	
5	*18.19*	*10.97* 7.22	*3.97*	*2.03* 1.88	*4.65*	*7.24*	*0.193*	*0.104* 0.089	*0.393*	*0.740*	2 hrs. narcosis.
6	11.00		4.12		2.70		0.190		0.574	0.916	
7	11.06		3.39		2.96						

Experiment VII. Nitrous oxide. The dog was phlorhizinized as usual and given some cold bathing. The figures on the chart begin with the 36th hour of preparation. Epinephrin was begun in period 4. The 2 hour nitrous oxide narcosis was conducted as in Experiments V and VI.

12 HR. PERIOD	GLUCOSE	NITROGEN	G:N	"EXTRA SUGAR"	GROSS SUGAR INCREASE	REMARKS
	gm.	*gm.*		*gm.*	*gm.*	
1	21.04	5.40	3.90			Phlorhizin 1 gm. in oil subcutaneously each 24 hrs.
2	22.09	5.78	3.82			
3	22.09	6.02	3.67			
4	53.52	6.07	8.82	30.76	31.43	Epinephrin 1 mg. subcutaneously each 6 hrs.
5	21.16	4.89	4.32	4.31		Epinephrin 1 mg. subcutaneously each 6 hrs.
6	22.83	5.50	4.14	2.75		Epinephrin 1 mg. subcutaneously each 6 hrs.
7	20.24	5.43	3.73	0.42		Epinephrin 1 mg. subcutaneously each 6 hrs.
8	*22.46*	*5.53*	*4.06*	*1.94**	*2.22**	N_2O narcosis 2 hrs.
9	19.61	5.52	3.55			Epinephrin as above.

* Based on figures of period 7.

Experiments VIII, IX, X, and XI. Acetaldehyde. The animals were given phlorhizin in the usual way plus epinephrin until G: N was constant. The acetaldehyde (Kahlbaum's) was given once pure (VIII) and three times in olive oil emulsion (IX, X, XI).

EXP. NO.	12 HR. PERIOD	GLUCOSE	NITROGEN	G:N	"EXTRA SUGAR"	GROSS SUGAR INCREASE	ACETONE BODIES*	REMARKS
		gm.	gm.		gm.	gm.		
VIII	1	11.15	3.81	2.93				Epinephrin 1 mg. subcutaneously each 3 hrs.
	2	10.94	3.68	2.96				Epinephrin 1 mg. subcutaneously each 3 hrs.
	3	10.94	3.80	2.87			0.625	Epinephrin omitted.
	4	10.88	3.76	2.90			0.603	" resumed.
	5	*11.31*	*3.73*	*3.03*	*0.42*	*0.40*	*0.603*	*Acetaldehyde 8.8 gm. subcutaneously. No epinephrin.*
	6	10.62	3.20	3.29	1.28	0.00	0.573	Epinephrin resumed as above.
IX	1	26.00	7.61	3.41				Epinephrin 1 mg. each 3 hrs., subcutaneously throughout the experiment.
	2	27.66	8.27	3.34				*Acetaldehyde 8.8 gm. subcutaneously.*
	3	26.10	8.16	3.20				
	4	*22.98*	*7.24*	*3.17*	*0.0*	*0.0*		
	5	21.90	4.13	5.30	8.35	0.0		
X	1	13.85	4.13	3.35				Epinephrin 1 mg. each 3 hrs.
	2	13.81	4.14	3.33				" omitted.
	3	13.03	3.90	3.37				" resumed.
	4	13.10	4.30	3.04				" omitted.
	5	12.58	4.10	3.07				" "
	6	*11.23*	*3.14*	*3.57*	*1.09*	*0.0*		*Acetaldehyde 8.8 gm. subcutaneously.*
	7	11.75	3.05	3.85	1.90	0.0		
XI	1	12.66	4.33	2.92				Epinephrin 1 mg. every 3 hrs. throughout the experiment.
	2	11.35	3.57	3.09				*Acetaldehyde 8.8 gm. subcutaneously.*
	3	12.63	4.30	2.93				
	4	*12.90*	*4.31*	*3.00*	*0.06*	*0.0*		
	5	6.90	1.15	6.00	3.47	0.0		

* Acetone, aceto-acetic, and β-hydroxy butyric acids were measured separately and reckoned in terms of β-hydroxy butyric acid.

Experiment XII. Phlorhizin alone, and plus epinephrin. The animal was phlorhizinized by the usual method until G: N was virtually constant, and then was epinephrinized. Note the rising G: N ratio during the last 12 hours with falling glycosuria similar to that seen in acetaldehyde experiments. In these periods the heart was failing.

6 HR. PERIODS	GLUCOSE	NITROGEN	G:N	"EXTRA SUGAR"*	REMARKS
	gm.	*gm.*		*gm.*	
1–5					30 hrs. urine discarded.
6–7	12.26	2.96	4.14		Hyphenated period numbers indicate figures obtained for 12 hr. periods divided by 2 for the sake of comparison with the succeeding 6 hr. periods.
8–9	12.31	3.07	4.00		
10–11	12.50	3.32	3.76		
12–13	12.75	3.48	3.66		
14	30.70	2.78	10.75	21.66	Epinephrin 1 mg. subcutaneously every 2 hrs. Animal failing in last 2 periods.
15	12.40	2.74	4.52	4.18	
16	8.44	2.73	3.09	0.0	
17	8.37	2.76	3.04	0.0	
18	8.50	2.60	3.27		
19	6.88	1.45	4.74		

* Reckoned on the basis that G:N equals 3.0, as in periods 16 and 17. This would imply that in periods 10–11 and 12–13 there had likewise been an excretion of 2.54 and 2.31 gm. of glycogen sugar respectively, provided that the ratios 3.09 and 3.04 are the true protein ratio for this dog. Similar late falls in the ratio have been noted when phlorhizin alone is given.

THE CHOLESTEROL METABOLISM OF THE HEN'S EGG DURING INCUBATION.

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The large amount of work which has been carried out dealing with the various aspects of the physiology and pathology of cholesterol, leaves many points to be cleared up before an accurate understanding of this complex question is possible. It has long been known that cholesterol occurs in the blood and tissues in two principal conditions; namely, free, and combined with the higher fatty acids as cholesterol oleate, palmitate, and stearate.[1] Little or nothing is known concerning the importance of the relative proportions of these two forms. Until the elaboration by Windaus[2] in 1910 of a quantitative method which made possible the determination of the two fractions separately, it was impossible to obtain any accurate information on the point. Since that time many interesting investigations have been carried out, principally upon the cholesterol of blood in diverse physiological and pathological conditions, by Gardner and coworkers,[3] Henes,[4] Havers,[5] and others.

There is as yet, however, no clear conception of the origin of the esters of cholesterol in the body, nor of their possible reconversion into free cholesterol. As a result of the work of Gardner and others, it is now pretty definitely established that

[1] K. Hürthle: *Ztschr. f. physiol. Chem.*, xxi, pp. 331–359, 1895.

[2] A. Windaus: *ibid.*, lxv, pp. 110–117, 1910.

[3] C. Dorée and J. A. Gardner: *Proc. Roy. Soc., Series B*, lxxx, pp. 212–239, 1908; lxxxi, pp. 109–128, 1909. M. T. Fraser and J. A. Gardner: *ibid.*, lxxxi, pp. 230–247, 1909; lxxxii, pp. 559–568, 1909–10. J. A. Gardner and P. E. Lander: *ibid.*, lxxxvii, pp. 229–236, 1913–14.

[4] E. Henes: *Deutsch. Arch. f. klin. Med.*, cxi, pp. 122–145, 1913.

[5] K. Havers: *ibid.*, cxv, pp. 267–289, 1914.

all the cholesterol of the body is taken in with the food, and that there is no synthesis of cholesterol in the body. Under normal dietary conditions the cholesterol of the food must be quite largely in the uncombined condition. It may be said that there are two sources of origin for it; namely, vegetable food and animal food. Since there is no synthesis by animals, the original substance must be one of the so called "phytosterols,"[6] substances which are probably of the same or nearly the same chemical formula as cholesterol itself, and which appear to be readily changed over by animals into true cholesterol. As far as I am aware, these phytosterols occur in plants only in the free or uncombined form. Hence the esters occurring in the animal body must be products of its own metabolism.

It seemed possible that a study of the cholesterol content of the egg during the period of incubation might throw some light on the relation of cholesterol and cholesterol esters in the life processes of animals, and that here, from the nature of the case, conditions would be ideal for studying this relation. The total cholesterol of eggs in varying stages of incubation has been investigated by Mendel and Leavenworth[7] and by Ellis and Gardner[8] in order to determine whether there might be an increase attributable to a synthesis. Their results were negative in that respect, their figures showing, however, a small but constant decrease when averages of a number of determinations have been made. This they attribute to experimental error, but it may be said in passing that there is some evidence[9] in support of the belief that bile acids are formed from cholesterol. Should this prove to be the case a slight decrease might be satisfactorily accounted for.

As regards the cholesterol ester content of chick embryos, Hanes[10] observed microscopically the presence of doubly re-

[6] A. Windaus and A. Hauth: *Ber. d. deutsch. chem. Gesellsch.*, xxxix, pp. 4378–4384, 1906. A. Windaus and A. Welsch: *ibid.*, xlii, pp. 612–616, 1909.

[7] L. B. Mendel and C. S. Leavenworth: *Am. Jour. Physiol.*, xxi, pp. 77–84, 1908.

[8] G. W. Ellis and J. A. Gardner: *Proc. Roy. Soc.*, *Series B*, lxxxi, pp. 129–132, 1909.

[9] J. Lifschütz: *Ztschr. f. physiol. Chem.*, xci, pp. 309–328, 1914.

[10] F. M. Hanes: *Jour. Exper. Med.*, xvi, pp. 512–526, 1912.

fractile fat droplets in the liver from the fifteenth day up to hatching, and also their persistence for about two weeks or more in the chick after hatching; but he notes their absence in the liver of the adult chicken. These doubly refractile droplets, becoming isotropic at about 40° to return again to the anisotropic condition upon cooling, are recognized as consisting for the most part of cholesterol esters. As far as I know this is the only record of such an observation, and since Hanes made no chemical analyses, it seemed desirable to do so. Hanes related the appearance of the esters to the process of calcification, and this question will be taken up more in detail after a consideration of the analytical results obtained.

Method of analysis.

The eggs used were all from the same breed of chicken. Beginning with the third day, an egg was broken every second day, the embryo cut into small pieces with scissors and placed together with the yolk sac and other contents, in alcohol. After twenty-four hours the alcohol was changed and later was replaced by ether. Following one change of ether, the solid material was dried and ground in a mortar. The combined alcoholic and ether extracts were evaporated to dryness and taken up in ether, the ether-insoluble part being rubbed up with dry sodium sulphate and added to the other solid material. This was now extracted for forty-eight hours in a Soxhlet apparatus, the extract transferred to a volumetric flask and made up to 100 cc. with ether. 10 cc. of this were used for the determination by the Windaus method[11] with a modification very similar to that of Fraser and Gardner.[12] The ether was evaporated off, about 30 cc. of alcohol were added, heated to boiling on the water bath and a 1 per cent solution of digitonin in 90 per cent alcohol was added. The alcohol was then evaporated off in a current of cold air from an electric fan, and the drying finished either by gentle warming, or by placing in a vacuum desiccator over sulphuric acid for a few hours if necessary. It was then taken up in ether, filtered through a tared filter paper, using as the tare a similar filter paper, which during the analyses was put through the same washing as the one containing the precipitate. This consists in washing thoroughly with ether, drying, and washing with boiling water. The two papers are finally dried at 105° to 110° for an hour and weighed. The ether washings are evaporated, the residue saponified with 2 gm. KOH in alcoholic solution for two hours on a water bath, then diluted with water and extracted with ether, using five 25 cc. portions. The com-

[11] Windaus: *loc cit.*

[12] Fraser and Gardner: *loc. cit.*

bined ether extracts are shaken out with two small portions of distilled water to remove small quantities of alkali which always contaminate the extract. The ether is then evaporated and the determination of cholesterol made as before. This final determination gives the cholesterol present originally as the ester.

The following protocol shows the results obtained.

DAY OF INCUBATION	FREE CHOLESTEROL	COMBINED CHOLESTEROL	FREE CHOLESTEROL IN TOTAL
	gm.	*gm.*	*per cent*
3	0.2159	0.0242	89.92
5	0.2470	0.0252	90.74
7	0.2643	0.0396	86.97
9	0.2985	0.0354	89.40
11	0.2249	0.0243	90.25
13	0.1993	0.0389	83.66
15	0.2035	0.0827	71.10
17	0.2115	0.0732	74.29
19	0.1789	0.0831	68.28
21	0.1575	0.1109	58.68
*2	0.1539	0.1026	60.00
*9	0.1289	0.0469	73.32

* Age of chick.

It is a recognized weakness of the Windaus method that in the presence of any considerable quantity of other lipoid material, values for the combined or ester fraction are too high. Since this condition obtains in these analyses, it may probably be safely assumed that the 10 to 12 per cent of ester found during the first two weeks is more than is really present, and that most of the cholesterol present is free.

While only one analysis was made in each case, the general trend of the process is very evident, consisting in little or no change up to about the end of the second week, and from then until the twenty-first day a rather sudden increase in the amount of combined cholesterol, causing a lowering of the proportion of the free to a minimum of about 60 per cent of the total. Following hatching of the chick there appears to be a slow decrease in the proportion of esters, but more analyses are necessary for definite conclusions as to this.

In general the results obtained bear out to a surprising degree the conclusions drawn by Hanes from microscopical observations. It should be noted, however, that by no means all of the esters

present are contained in the liver, nor is the cholesterol of the liver all in the combined form. The livers of five twenty day embryos were analyzed together, yielding 0.0179 gram of free cholesterol and 0.0516 gram of combined cholesterol, while in the combined bodies of the five embryos, with the yolk sac, still partially unabsorbed, there must have been about 0.3 gram of combined cholesterol.

As to the significance of this rather sudden production of cholesterol esters during the final week of incubation, it was the idea of Hanes that they were connected with the process of calcification in the following way. Plimmer and Scott[13] showed by analyses of the phosphorus compounds of the incubated egg, that, corresponding to the period of calcification, *viz.*, from the fourteenth day on, there was a sudden drop in the amount of lecithin or "phosphorized fats" which had previously remained pretty constant. Since the decrease in the vitellin phosphorus was gradual and fairly constant during the whole three weeks of incubation, it seemed plain that the phosphorus necessary for calcification came from a breaking down of the lecithin. Hanes believes that in this breaking down, which he suggests may take place in the liver, some of the liberated fatty acids, which might otherwise exert a toxic action, are combined with the cholesterol to form non-toxic substances, the cholesterol esters.

This theory fits the known facts well. It is at once evident, however, that approximately 0.10 gram of cholesterol is inadequate to combine with the fatty acids liberated in the breaking down of 0.7 to 0.9 gram of lecithin. Still it may be readily believed that the greater part of the acid could be oxidized or recombined as a neutral fat with glycerol, only a small part being left unprovided for, to be taken care of by the cholesterol.

As to the ultimate fate of these accumulations of esters, little can be said with assurance. It is certain that there is a decrease in the proportion of esters, beginning soon after hatching. Röhmann,[14] Schultz,[15] Cytronberg,[16] and Gardner and Lander[17] have

[13] R. H. A. Plimmer and F. H. Scott: *Jour. Physiol.*, xxxviii, pp. 247–253, 1909.

[14] F. Röhmann: *Berl. klin. Wchnschr.*, xlii, pp. 1993–1994, 1912.

[15] J. H. Schultz: *Biochem. Ztschr.*, xlii, pp. 255–261, 1912.

[16] S. Cytronberg: *ibid.*, xlv, pp. 281–283, 1912.

[17] Gardner and Lander: *Biochem. Jour.*, vii, pp. 576–595, 1913.

reported the splitting of cholesterol esters on autolysis of liver, or mixtures of liver and blood from various animals, presumably by the action of some specific enzyme. Rothschild[18] believes that he has microscopical evidence for the splitting of these esters in the Kupffer cells of the liver. We have been unable to obtain results suggesting enzyme action in the liver either of chicks just hatching, or of adult chickens, but propose to investigate this point further.

There seems, then, to be evidence that cholesterol is, at least in part, a protective substance, having for its function the power to combine with and "detoxify" the fatty acids, substances which when introduced into the circulation exert a marked toxic action through hemolysis.

It is worthy of note in this connection that cholesterol can also unite with the various saponins, substances which are also hemolytic. Upon this union the Windaus method depends for estimation of cholesterol, the compound with digitonin (a saponin) being of definite composition, and very difficultly soluble in alcohol, from which it crystallizes.

CONCLUSIONS.

1. The cholesterol of the newly laid hen's egg is practically all in the free condition.

2. During the period of incubation this condition obtains until about the thirteenth day, from which time there is a gradual esterification until, at the time of hatching, over 40 per cent of the cholesterol present is in the form of esters.

3. The esterifying cholesterol may function as a detoxifying substance, with which the toxic fatty acids, set free from lecithin during the latter stage of embryonic development, combine to form harmless esters.

[18] M. A. Rothschild: *Proc. N. Y. Path. Soc.*, xiv, pp. 159–171, 1914.

THE ESTIMATION OF LIPOID AND ACID-SOLUBLE PHOSPHORUS IN SMALL AMOUNTS OF SERUM.[1]

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In previous publications[2] the author has shown that the phosphorus compounds of blood and serum may be divided into three classes: (1) lipoid; (2) a form soluble in a mixture of picric and acetic, or hydrochloric acids, and probably inorganic in nature; (3) protein. The last is present only in small quantities in blood. In serum, if present at all, it is negligible in a determination of the other fractions. Although the fact was not emphasized at the time, it is evident from the experiments then reported that all the lipoid phosphorus is carried down in the precipitate produced by the addition of blood or serum to picric-acetic acid mixture.

These experiments may be briefly summarized here. Blood or serum was treated with acetone and the precipitate extracted with boiling acetone. The combined extracts were evaporated and the lipoids precipitated with chloroform and dilute hydrochloric acid.[3] The filtrate from the lipoids contained only traces of phosphorus. The residue from the acetone extract was extracted with boiling alcohol and then with boiling ether. Only traces of phosphorus were found in the combined extracts. The resulting residue was treated with a saturated solution of picric acid in 0.4 per cent hydrochloric acid. The amount of phosphorus in a measured portion of the extract was estimated, and the total acid-soluble phosphorus calculated. Added inorganic phosphate was quantitatively recovered in the acid extract. Precipitation of the proteins and lipoids of the blood with a solution containing 1 per cent each of acetic and pic-

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[1] Reported to the Society for Experimental Biology and Medicine, January 20, 1915. *Proceedings*, xii, p. 71, 1915.

[2] I. Greenwald: this *Journal*, xiv, p. 369, 1913; *Am. Jour. Med. Sc.*, cxlvii, p. 225, 1914.

[3] W. Koch and H. S. Woods: this *Journal*, i, p. 206, 1905–06.

ric acids yielded the same amount of acid-soluble phosphorus as the preceding treatment. Added inorganic phosphate was also completely recovered.

This procedure has now been combined with the Neumann method of oxidation and the method of Pouget and Chouchak[4] for the determination of minute quantities of phosphoric acid for the purpose of estimating the amount of lipoid and other forms of phosphorus in small quantities of serum (1 cc. and less). This method apparently depends upon the formation of strychnine phosphomolybdate in a finely suspended condition. Although not apparent until placed in the colorimeter, the opalescent liquid has a yellow color, the depth of which is, within wide limits (from a concentration of two-thirds to over twice the standard), proportional to the amount of phosphoric acid present. This method has been in use for some time and has given satisfactory results, duplicate determinations agreeing closely with one another and with the values obtained by a modified Neumann method.[5] The error is about ± 2 per cent. A few comparisons with the Neumann method are given in Table I.

TABLE I.

Amount of phosphorus in various specimens as determined by a modified Neumann method and by the micro method.

	NEUMANN METHOD	MICRO METHOD
	per cent	*per cent*
I	0.0138	0.0136
II	0.0243	0.0239
III	{0.1198 0.1152	{0.1176 0.1170

The procedure is as follows: 1 cc. of serum is measured into a 10 cc. volumetric flask containing about 8 cc. of the picric-acetic acid mixture, and the volume made up to the mark with the same solution. The serum must be free from hemoglobin, as otherwise it contains enough inorganic phosphates derived from the erythrocytes to vitiate the analyses. After standing

[4] I. Pouget and D. Chouchak: *Bull. soc. chim.*, series 4, v, p. 104, 1909; ix, p. 649, 1911.

[5] Greenwald: this *Journal*, xiv, p. 369, 1913.

for two hours with occasional shaking, the mixture is filtered through a 5.5 cm. No. 589 black ribbon S and S filter paper into a standard 10 cc. graduated cylinder. After reading the volume of the filtrate, it is transferred to a Jena test-tube, 200 by 20 mm. A few small glass beads are added and the liquid is boiled down to a volume of about 1 cc.; 1 cc. of concentrated sulphuric acid is added and the mixture is heated until all the picric acid has been distilled off. (The sides of the tube should be heated to insure the complete removal of the picric acid.) The oxidation is completed by the addition of one or two drops of concentrated nitric acid and subsequent heating. The residue and the filter paper are transferred to another test-tube, 1 cc. of sulphuric acid is added, and the oxidation is carried out in the usual manner, using as much nitric acid as is necessary. After cooling, the acid is diluted and almost neutralized with 10 per cent sodium hydroxide (from sodium) solution. The solution is then made up to a convenient volume, generally 50 cc., and aliquot portions, generally 20 and 10 cc., are taken for the determinations. These amounts are measured into 50 cc. volumetric flasks, 5 cc. dilute nitric acid (35 cc. concentrated acid diluted to 100 cc.) are added, and the mixture is diluted to about 45 cc. After thorough mixing, 2 cc. of the reagent of Pouget and Chouchak[6] are added, the volume is made up to 50 cc., and the liquid mixed thoroughly. At the same time 5 cc. of a solution containing 0.0131 mg. of phosphorus (0.03 mg. P_2O_5) are measured into another flask and similarly treated. After standing for at least twenty minutes, the colors of the liquids are compared in a Dubosq colorimeter. It is best to set the standard at 80 mm. Good results may be obtained at 40 mm., but there is then more difficulty in comparing the colors. In calculating the results, proper correction is made for the volume of solution adhering to the protein-picrate precipitate.

The results of a number of determinations in specimens of serum obtained from hospital patients and from normal individuals

[6] *Preparation of reagent.* (A) Dissolve 95 gm. of molybdic acid and 30 gm. of dry sodium carbonate in 500 to 600 cc. of warm H_2O; after cooling, add 141 cc. of concentrated nitric acid and dilute to 1000 cc. (B) Dissolve 2 gm. of strychnine sulphate in 90 cc. of warm water and dilute to 100 cc. For use, add 1 cc. of B to 10 cc. of A, filter, and use immediately.

are summarized in Tables II and III. The first column of figures represents the amount of non-protein nitrogen in the serum, as determined by the Folin-Denis[7] procedure. The amounts of acid-soluble and of lipoid phosphorus appear in the

TABLE II.

Normal and pathological sera.

NAME	DIAGNOSIS	NON-PROTEIN NITROGEN PER 100 CC.	PHOSPHORUS PER 100 CC.			
			Acid-soluble	Lipoid	Sum	Total
		mg.	*mg.*	*mg.*	*mg.*	*mg.*
W.	Normal		4.46	6.64	11.10	11.33
J. S.	"		1.97	7.28	9.25	9.54
C. E. A.	"		3.77	10.72	14.49	13.60
B.	"		4.14	9.12	13.26	13.65
I. G.	" 4 hrs. after breakfast	21.3	3.99	9.31	13.30	13.90
"	" " " "	21.2	2.10	11.50	13.60	13.76
"	" 2 hrs. after lunch	26.1	6.10	10.90	17.00	16.10
"	" " " "	20.6	6.06	12.95	19.01	19.65
M. E.	" 6 hrs. after breakfast	22.0	4.50	9.08	13.58	13.88
"	" 2½ hrs. after lunch	21.2	6.80	11.74	18.54	18.75
H. E.	" 6 hrs. after breakfast	23.7	4.69	10.11	14.80	15.52
"	" 2 hrs. after lunch	21.1	3.45	8.58	12.03	11.03
F. C.	Chronic bronchitis, morphinism	28.3	3.97	7.32	11.29	12.55
R.	Pernicious anemia		4.60	7.54	12.14	12.27
K. F.	Ulcer of foot, tabes (?)	17.8	4.73	7.48	12.21	11.40
M. C.	Hypertension (260 mm.), hemiplegia	27.7	4.52	11.01	15.53	15.48
M. B.	Chronic endocarditis, pneumonia	18.0	6.75	7.91	14.66	13.80
H. B.	Pneumonia	29.8	3.43	10.61	14.04	15.03
F.	Polycythemia	26.2	4.41	11.77	16.18	14.74
H. W.	Aortic aneurysm, chronic cardiac insufficiency	19.3	3.28	12.00	15.28	15.94
T. B.	Influenza, chronic endocarditis	22.9	2.00	8.23	10.23	10.26
L. B.	Chronic endocarditis, pneumonia	20.4	4.01	4.81	8.82	8.99

following two columns. The figures in the fourth column represent the sum of those in the two preceding, while those in the fifth and last column show the amount of total phosphorus in the serum, as determined directly in another portion. These

[7] O. Folin and W. Denis: this *Journal*, xi, p. 527, 1912.

results are in full agreement with those previously obtained with large amounts of serum by direct extraction of the lipoids followed by treatment with hydrochloric-picric acid solution. These results have already been reported.[8]

It may be well to compare the figures with those reported by others. Peritz,[9] Bornstein,[10] and Beumer and Bürger[11] all obtained much smaller values for the amount of lipoid phosphorus in the serum than have been obtained by the present writer. The disagreement appears to be due entirely to differences in the method of extracting the lipoids. All the authors mentioned dried the serum, with or without the addition of sand, and then extracted the dry residue with the usual solvents. It is probable that, under these conditions, extraction is not complete. The dry protein surrounds the lipoids with a layer of insoluble material that is only very slowly penetrated by the solvent. Moreover, chemical changes occurring during the drying of the serum may render some of the lipoid phosphorus insoluble in the solvents employed. Klein and Dinkin[12] treated the serum with anhydrous sodium sulphate and extracted the mixture with alcohol and ether. This extract was evaporated and the residue treated with ether. Phosphorus was determined in the ethereal solution and also, occasionally, in the residue. Their results seem to indicate that extraction was not complete and that, in the subsequent evaporation, some of the phospholipins were decomposed. Lesser[13] treated the serum with absolute alcohol, at first in the cold, then repeatedly at 60°. The extracts were evaporated in a current of air, dissolved in a mixture of alcohol and ether and again evaporated, finally dissolved in absolute ether, and filtered. Phosphorus was determined in the filtrate. The results obtained were a little lower than those reported in this paper. In only two cases have we obtained less than 7 mg. of lipoid phosphorus per 100 cc. of serum, whereas, out of seventy-seven specimens examined, Lesser found twenty with less than this amount. Correspondingly, Lesser's average is a little less than 9 mg., whereas the average of the results reported in Table II is almost 10 mg. per 100 cc.

Taylor and Miller,[14] using their method of estimating phosphorus find from 4 to 6 mg. of lipoid phosphorus per 100 cc.[15] The presence of inor-

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[8] Greenwald: *Am. Jour. Med. Sc.*, cxlvii, p. 225, 1914.

[9] G. Peritz: *Ztschr. f. exper. Path. u. Therap.*, v, p. 607, 1908–09.

[10] A. Bornstein: *Monatschr. f. Psychiat. u. Neurol.*, xxv, p. 160, 1909; *Ztschr. f. d. ges. Neurol. u. Psychiat.*, vi, p. 605, 1911.

[11] H. Beumer and M. Bürger: *Ztschr. f. exper. Path. u. Therap.*, xiii, p. 343, 1913.

[12] W. Klein and L. Dinkin: *Ztschr. f. physiol. Chem.*, xcii, p. 302, 1914.

[13] F. Lesser: *Arch. f. Dermat. u. Syph.*, cxiii, p. 609, 1912.

[14] A. E. Taylor and C. W. Miller: this *Journal*, xviii, p. 214, 1914.

[15] A personal communication from Dr. Taylor indicates that the low values obtained were due to incomplete extraction.

ganic phosphate is denied. It is possible that there is no inorganic phosphate in serum, but what is termed acid-soluble phosphorus in this paper can only be present as inorganic phosphate or closely related compounds, such as glycero-phosphoric or inosinic acids. It seems certain that it is not the latter, for experiments carried out in this laboratory have shown that the serum of dogs or horses does not contain purine nitrogen corresponding in amount to the acid-soluble phosphorus, on the assumption that this is present as inosinic acid.

In three out of four experiments there was a marked increase in the amount of acid-soluble phosphorus in the serum shortly after luncheon, containing meat. In the fourth experiment the subject, H. E., also ate a fruit salad and drank water freely between luncheon and the time of drawing the blood. It is possible that the salad, owing to the excess of alkaline ash constituents, may have served to prevent the increase in the amount of acid-soluble phosphorus in the serum. The possible diluting effect of the water ingested is also to be considered. It would seem from the large variations found that the content of this form of phosphorus in the serum may be largely dependent upon the character of the diet and the stage of digestion. An attempt was made to eliminate this factor in the other experiments by taking all specimens at least four hours after the last meal, but, as the patients were not under strict control, we cannot be certain that this was always attained.

The amount of lipoid phosphorus, though varying widely in different individuals, is comparatively constant for the same individual. It does not appear to be increased during the absorption of fat; at least, milky sera were not distinguished by their high content of lipoid phosphorus.

The cases reported in Table III are arranged roughly in the order of the content of non-protein nitrogen in the serum. As a rule, those patients whose serum shows little or no increase in the non-protein nitrogen also have comparatively slight retention of acid-soluble phosphorus. A marked retention of nitrogen is usually accompanied by an increase in the acid-soluble phosphorus. But these do not parallel each other. For instance, the serum of L. P. contained only 31 mg. of nitrogen per cc., but as much as 20.2 mg. of phosphorus. This patient died in coma two days later. The serum of A. C. contained 111 mg. of nitro-

gen, but only 6.76 mg. of phosphorus per 100 cc. Again, in the case of M. L. and J. M. there are great changes in the non-protein nitrogen of the serum of the same individual, with comparatively little change in the amount and nature of the

TABLE III.

Nephritic sera.

NAME	NON-PROTEIN NITROGEN PER 100 CC.	PHOSPHORUS PER 100 CC.			
		Acid-soluble	Lipoid	Sum	Total
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
H. W.	19.3	3.25	9.71	12.96	14.77
M. R.	30.7	3.60	12.60	16.20	15.60
"	20.0	5.79	13.38	19.17	18.38
"	20.3	5.32	12.95	18.27	20.12
"	23.9	5.90	15.09	20.99	19.70
J. W.	29.2	3.99	13.24	17.23	16.75
G. W.	29.2	4.46	8.58	13.04	12.26
J. McK.	25.0	5.79	8.75	14.54	15.73
T. G.	26.1	6.03	10.92	16.95	18.25
E. L.	24.9	6.16	10.44	16.60	15.55
F. V.	35.6	4.82	8.68	13.50	14.17
L. P.	31.4	20.2	12.90	33.1	33.2
M. E.	43.2	5.39	11.81	17.20	17.11
J. H.	42.8	12.30	36.20	48.5	49.4
E. H.	59.5	6.56	6.69	13.25	12.87
H. W.	111.	12.52	11.11	23.63	23.72
"	67.4	6.17	11.41	17.58	17.70
A. C.	111.	6.76	10.20	16.96	
M. D.	103.	15.1	16.4	31.5	33.2
"	103.	21.1	16.7	37.8	35.6
M. L.	150.	12.72	8.25	20.97	20.80
"	255.	12.71	8.21	20.92	20.63
J. M.	133.	15.72	7.78	23.50	23.21
"	194.	16.29	9.90	26.19	29.40
N. K.	200.	12.52	7.80	20.32	20.74

phosphorus. Conversely, in the case of M. D. the amount of non-protein nitrogen in the two specimens was the same, while the amount of acid-soluble phosphorus differed greatly.

It would also appear that, in nephritis, the amount of lipoid phosphorus may be increased. Whereas in the other sera the

highest amount of lipoid phosphorus was 13 mg. per 100 cc., this was frequently surpassed in the nephritic sera, in one of which the astonishing amount of 36.2 mg. was found.

It is with great pleasure that I acknowledge my indebtedness to the house staff of Roosevelt Hospital, and particularly to the pathologist, Dr. Mortimer Warren, for their aid in obtaining material.

SUMMARY.

A method for the estimation of lipoid and of acid-soluble phosphorus in small amounts of serum is described. It is probably applicable to other tissues. The amount of phosphorus not precipitated by a dilute solution of acetic and picric acids varies, in normal individuals, between 2 and 6 mg. per 100 cc. of serum. The amount appears to be dependent on the character of the diet and the stage of digestion. The amount of lipoid phosphorus lies between 5 and 13 mg. (usually between 7 and 13 mg.) per 100 cc. of serum. It is apparently less subject to variation in the same individual than is the amount of acid-soluble phosphorus. In nephritis, there is evidence of retention of inorganic phosphate, and also, in some cases, a certain increase in the amount of lipoid phosphorus in the serum.

ON THE SEVERAL FACTORS OF ACID EXCRETION IN NEPHRITIS.

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In the course of our experimental studies[2] of the regulatory excretion of acid, two pathological conclusions have been reached: first, that the urinary concentration of ionized hydrogen is, in a statistical sense, increased in the various forms of nephritis; and, secondly, that such pathological states are frequently marked by a condition of acidosis. The latter deduction appears, at any rate, inevitable from the fact that when alkali is administered it is in these cases retained by a kidney capable of the rapid elimination of an excess of alkali. This view also agrees with numerous other observations upon nephritis. Meanwhile we have investigated in the normal individual the shares of the several factors of acid excretion in the regulatory process. Considering the great variability of physiological activities in general, and the many complicating influences which, in particular, have been revealed in the analogous regulation of body temperature, it is probably too much to regard these latter results as providing more than a very rough outline of the process. They have,

[1] Henry P. Walcott Fellow in Clinical Medicine, Harvard Medical School.

[2] L. J. Henderson and W. W. Palmer: On the Intensity of Urinary Acidity in Normal and Pathological Conditions, this *Journal*, xiii, p. 393, 1912–13; On the Extremes of Variation of the Concentration of Ionized Hydrogen in Human Urine, *ibid.*, xiv, p. 81, 1913. Palmer and Henderson: Clinical Studies on Acid Base Equilibrium and the Nature of Acidosis, *Arch. Int. Med.*, xii, p. 153, 1913. L. H. Newburgh, W. W. Palmer, and L. J. Henderson: A Study of Hydrogen Ion Concentration of the Urine in Heart Disease, *ibid.*, xii, p. 146, 1913. Henderson and Palmer: On the Several Factors of Acid Excretion, this *Journal*, xvii, p. 305, 1914.

nevertheless, seemed to us not inadequate as a basis for pathological comparisons, and, taking them as such, we have now to report our observations upon these factors in nephritis.

The procedure in this research is similar to that which we have previously adopted. We have not sought to carry out very carefully controlled observations, involving systematic studies of total metabolism and the like, but, abandoning the unquestionable advantages of such a method, have sought through the use of quick methods in large numbers of cases the advantages of statistical treatment.[3] Nevertheless we have made independent simultaneous investigations on the effect of diet. These

[3] It seems to us that Blatherwick's recent dissent from certain of our views as a result of his own excellent studies of the influence of diet upon the excretion of acid (*Arch. Int. Med.*, xiv, p. 409, 1914), is due to a misconception of what we are thus undertaking. For example, in the matter of a rough parallelism between volume of urine and the intensity of its acidity we have sought merely to point out a statistical result, a result which, only if it can be explained by the recognition of some physiological connection, will become significant, but which it is futile to object to on the ground that more restricted cases (*i.e.*, cases in which the diet is controlled) do not reveal it. And, of course, it neglects the whole idea of statistics and the basis of statistical theory to point out that individual observations are not in accord with the average. It is equally futile to assume a direct causal connection because of such observations; but this we have not done. Are not men taller then women statistically, although some women are taller than some men individually? It is our purpose to consider the use of statistical methods in the study of renal function in a future paper.

Another point in which Blatherwick disagrees with us concerns the rôle of ammonia in regulating the reaction of the blood. Here the difficulty appears to be that we did not make our meaning clear. As far as we are aware nobody has, for years past, doubted that the excretion of ammonia is one essential factor in regulating the reaction of the body. What we believe, however, is that the true regulation is to be found in the control of the production of ammonia and that in certain cases this is not correlated with the other regulatory process in the kidney. This conclusion depends upon the fact that in a considerable number of normal cases no correlation between the hydrogen ion concentration and the ammonia content of the urine was to be observed. We can only account for this fact upon the assumption that the final adjustment of the alkalinity of the body, *viz.*, the regulation through the activity of the kidney of the hydrogen ion concentration in the blood (we did not say in the body generally) depends almost wholly upon the acid excretion. The only objection that we can imagine to this statement is that, without any investigation at all, it is self-evident.

prove that variations of diet cannot significantly influence the results upon which our present conclusions are based. The gain which we have obtained by following this plan appears to us to justify the carefully considered original decision, but for the present we postpone a discussion of this question.

Our complete observations now extend to forty-four cases of nephritis and involve analyses of the daily excretion for 311 days. These data are to be compared with our earlier observations upon sixteen normal individuals over a period amounting in all to 122 days. In the course of these studies we have in all cases estimated the hydrogen ion concentration, acid excretion, ammonia excretion, and volume, as well as, at times, certain other quantities which do not now concern us. From these values have been calculated the amount of total acid excretion, A, and of R, the ratio of acid excretion to ammonia excretion. These topics are all discussed in an earlier paper.

In the present paper the following symbols are used: H, representing the hydrogen ion concentration as expressed by the negative logarithm thereof (P_H^+ of Sörensen); A, representing the acid excretion, or, in other words, the amount of acid in the urine in excess of that combined with the urinary bases as they existed in the blood, this quantity being expressed in cubic centimeters of decinormal solution *per diem;* NH_3, the total urinary ammonia, expressed in like manner; TA, the sum of $A + NH_3$; V, the volume of urine eliminated in twenty-four hours; R, the ratio, $\frac{A}{NH_3}$.

In the following table the data are collected, the cases being arranged in the order of the average values of R's (the reason for this arrangement is explained below). All estimates have been made in duplicate with close agreement in the results.

The reason for arranging the cases in the above order will be at once evident from an examination of the table; for thus arranged the cases fall into two quite distinct groups (except cases XV and XVI, in which observations are too few). The one group manifests values of R calculated for the total quantities of A and NH_3 in the period studied equal to or greater than 1.6, and the other values less than 1.3. The unavoidable impression that this is no mere chance is greatly strengthened by the invariable highness of the daily values of R in all the cases of Group

TABLE I.

CASE	V	H	A	NH3	TA	R
I R* = 3.95 Chronic nephritis, uremia	1075	5.3	200	69	269	2.90
	1075	5.3	228	57	285	4.00
	1075	5.3	200	43	243	4.65
	1150	5.3	240	51	291	4.70
Mean...........................	1094	5.3	217	55	272	4.06
II R = 3.33 Chronic nephritis, uremia	1050	5.3	142	50	192	2.84
	1850	5.5	278	81	359	3.44
	1300	5.7	172	48	220	3.58
	1375	5.7	181	58	239	3.12
	1250	5.7	210	57	267	3.69
Mean...........................	1365	5.6	196	59	255	3.33
III R = 2.97	3150	5.4	430	181	611	2.38
	2020	5.3	380	110	490	3.45
	1140	5.1	390	76	466	5.13
Chronic glomerulonephritis, uremia	1840	6.0	202	98	300	2.06
	2610	5.5	460	135	595	3.40
	2700	5.4	270	100	370	2.70
	2500	5.5	315	125	440	2.52
Mean...........................	2280	5.5	350	118	468	3.09
IV R = 2.74 Chronic glomerulonephritis, uremia	1710	5.0	240	100	340	2.40
	2250	5.0	308	100	408	3.08
Mean...........................	1980	5.0	274	100	374	2.74
V R = 2.07 Renal arteriosclerosis, uremia	1465	5.0	284	128	412	2.22
	1230	5.0	246	128	374	1.92
Mean...........................	1346	5.0	265	128	393	2.07
VI R = 1.94 Acute nephritis, mild uremia	1540	5.1	415	270	685	1.54
	1840	5.0	390	220	610	1.77
	1320	5.3	317	164	481	1.93
	1380	5.3	373	163	536	2.28
	1660	4.9	480	202	682	2.37
Mean...........................	1548	5.1	395	204	599	1.98

* The R under the case number is obtained by dividing the mean values of A by the mean values of NH_3, while the mean R in the column R is the average of the various R's.

TABLE I—*Continued.*

CASE	V	H	A	NH_3	TA	R
VII	1400	7.0	84	75	159	1.12
R = 1.93	600	5.5	114	75	189	1.52
Chronic glomerulonephritis, uremia	900	5.4	173	61	234	2.84
	1625	6.0	204	87	291	2.34
Mean	1131	5.9	144	75	219	1.95
VIII	1020	4.9	293	157	450	1.87
R = 1.89	800	4.9	244	155	399	1.57
	960	4.9	236	132	368	1.79
	860	4.9	267	137	404	1.95
Chronic glomerulonephritis, mild	940	4.8	298	146	444	2.04
uremia	1620	4.9	324	189	513	1.71
	1565	4.9	455	183	638	2.48
	1475	5.0	394	190	584	2.07
	1460	5.0	344	220	564	1.56
Mean	1189	4.9	316	168	484	1.89
IX	1970	5.3	324	243	567	1.33
R = 1.86	1500	5.1	300	153	453	1.96
	3710	5.1	334	195	529	1.71
Acute nephritis, mild uremia	2360	5.1	275	70	345	3.93
	3315	5.1	300	165	465	1.82
Mean	2571	5.1	307	165	472	1.86
X	660	5.4	115	100	215	1.15
R = 1.80	1160	4.8	266	155	421	1.72
Chronic glomerulonephritis, uremia	740	5.1	155	85	240	1.82
	1100	5.5	234	136	370	1.72
	900	4.8	193	58	251	3.32
Mean	912	5.1	193	107	300	1.95
XI a	1900	5.3	380	370	750	1.03
R_a = 1.65	2370	5.1	455	270	725	1.69
	3500	5.1	670	350	1020	1.91
Syphilitic nephritis	3300	5.1	660	358	1018	1.85
	2650	5.0	570	268	838	2.12
	4000	5.1	500	330	830	1.52
	3750	5.1	620	400	1020	1.55

TABLE I—*Continued.*

CASE	V	H	A	NH_3	TA	R
XI b	1620	5.7	254	165	419	1.54
R_b = 1.79	1740	5.5	335	162	497	2.06
	2000	5.3	400	206	606	1.94
R = 1.72	2100	5.1	425	244	669	1.74
	2500	5.5	510	317	827	1.61
	2800	5.0	475	262	737	1.81
	2520	5.1	400	245	645	1.63
	3050	5.4	455	255	710	1.78
	2500	5.3	425	222	647	1.91
	2750	5.3	500	276	776	1.81
	2450	5.3	465	245	710	1.90
Mean	2640	5.2	472	275	747	1.74
XII a	1260	5.0	430	310	740	1.39
R_a = 1.49	1080	5.0	370	266	636	1.39
	1200	5.3	430	250	680	1.72
	950	5.3	360	210	570	1.72
Chronic glomerulonephritis, mild	1200	5.3	425	258	683	1.65
uremia	1000	5.1	294	231	525	1.27
	1080	5.0	388	284	672	1.37
	700	5.0	282	148	430	1.90
	810	4.8	284	190	474	1.50
	700	5.0	238	200	438	1.19
XII b	770	5.6	320	227	547	1.41
R_b = 1.86	560	5.5	279	171	450	1.63
	540	5.3	237	137	374	1.73
R = 1.63	800	5.4	277	169	446	1.64
	600	5.3	259	104	363	2.50
	620	5.2	264	154	418	1.72
	630	5.5	225	137	362	1.64
	1000	5.3	349	137	486	2.54
	1000	4.8	335	132	467	2.54
Mean	868	5.2	318	195	513	1.71
XIII	2140	5.1	445	268	713	1.66
R = 1.62	1060	5.3	185	171	356	1.08
	1040	5.1	248	152	400	1.63
Chronic glomerulonephritis, mild	1600	5.0	330	235	565	1.40
uremia	1500	5.0	262	176	438	1.49
	1875	5.1	312	207	519	1.51
	1300	5.1	300	195	495	1.54
	1160	5.1	470	234	704	2.00
	1600	5.1	337	160	497	2.10
	1500	5.0	290	166	456	1.75
Mean	1478	5.1	318	196	514	1.62

TABLE I—*Continued.*

CASE	V	H	A	NH_3	TA	R
XIV	930	5.5	317	166	483	1.91
R = 1.58	1050	5.3	210	192	402	1.09
Chronic glomerulonephritis, uremia	740	5.5	154	77	231	2.00
	1000	5.3	185	114	299	1.62
Mean	930	5.4	217	137	354	1.65
XV	1080	6.0	230	151	381	1.52
R = 1.46	960	5.8	270	191	461	1.41
Chronic glomerulonephritis	975	5.8	347	237	584	1.46
Mean	1005	5.9	282	193	475	1.46
XVI	4120	5.0	515	375	890	1.37
R = 1.44	3300	5.0	410	270	680	1.52
Chronic interstitial nephritis						
Mean	3710	5.0	463	322	785	1.44
XVII	1780	5.0	400	318	718	1.26
R = 1.24	1430	5.0	422	284	706	1.48
	1610	4.9	480	400	880	1.20
Chronic glomerulonephritis	1500	4.9	442	350	792	1.26
	1350	4.9	405	387	792	1.05
Mean	1534	4.9	430	348	778	1.25
XVIII	1200	5.3	205	240	445	0.86
R = 1.16	725	5.4	124	87	211	1.42
	1200	4.8	515	128	643	4.01
	950	5.1	300	145	445	2.06
	1260	4.8	372	450	822	0.83
	800	4.8	204	135	339	1.51
Chronic interstitial nephritis	800	5.3	200	210	410	0.95
	975	4.8	230	200	430	1.15
	1200	4.8	330	365	695	0.90
	860	5.1	204	286	490	0.71
	960	5.0	270	300	570	0.90
Mean	994	5.0	269	231	500	1.39
XIX	630	5.1	193	230	423	0.84
R = 1.09	650	5.0	254	180	434	1.41
Chronic glomerulonephritis						
Mean	640	5.1	224	205	429	1.13

TABLE I—*Continued.*

CASE	V	H	A	NH_3	TA	R
XX	840	5.4	168	200	368	0.84
R = 1.08	1180	5.1	270	294	564	0.92
Chronic nephritis	820	5.3	158	160	318	0.99
	900	5.3	328	200	528	1.64
Mean	935	5.3	231	213	444	1.10
XXI	1300	6.7	267	163	430	1.64
R = 1.06	1925	6.5	338	280	618	1.21
	1550	5.7	260	280	540	0.89
Cardiorenal disease	1520	5.3	220	334	554	0.65
	950	5.1	380	340	720	1.12
	1140	5.7	296	270	566	1.10
Mean	1397	5.8	293	278	571	1.10
XXII	360	5.0	113	154	267	0.73
R = 1.06	740	5.0	148	174	322	0.85
	800	5.7	86	148	234	0.58
	3000	5.1	320	257	577	1.25
	2700	5.3	164	250	414	0.66
	1700	5.0	224	145	369	1.54
Cardiorenal disease	1500	5.0	218	185	403	1.18
	1800	5.0	300	330	630	0.91
	1200	4.7	185	288	473	0.64
	1260	4.7	445	166	611	2.68
	1300	4.7	173	152	325	1.14
Mean	1487	5.0	216	204	420	1.11
XXIII	750	5.0	490	425	915	1.15
R = 1.05	630	5.1	325	246	571	1.32
Subacute glomerulonephritis	1725	5.1	600	680	1280	0.88
Mean	1035	5.1	472	450	922	1.12
XXIV	300	5.7	108	192	300	0.56
R = 0.98	200	5.8	56	60	116	0.93
	650	5.3	316	370	686	0.86
Subacute glomerulonephritis	500	5.3	235	336	571	0.70
	400	5.1	246	183	429	1.34
	750	5.1	440	320	760	1.37
	270	5.0	156	122	278	1.28
Mean	440	5.7	222	226	448	1.01

TABLE I—*Continued.*

CASE	V	H	A	NH_3	TA	R
XXV	400	5.3	286	272	558	1.05
R = 0.98	410	5.4	242	268	510	0.90
Chronic glomerulonephritis						
Mean	405	5.4	264	270	534	0.98
XXVI	780	5.0	340	370	710	0.92
R = 0.97	950	5.6	323	395	718	0.82
Nephritis (arteriosclerotic?)	940	5.4	357	290	647	1.23
Mean	890	5.3	340	352	692	0.99
XXVII	1260	5.5	183	346	529	0.53
R = 0.97	1880	4.8	395	293	688	1.35
	1790	4.7	532	330	862	1.61
	1280	4.7	433	495	928	0.87
Chronic glomerulonephritis	1880	4.7	402	462	864	0.87
	1270	5.1	291	300	591	0.97
	2140	4.7	460	542	1002	0.85
Mean	1643	4.9	385	395	780	1.01
XXVIII	1260	5.3	366	341	707	1.06
R = 0.97	1300	5.3	525	530	1055	0.99
	560	5.0	350	343	693	1.02
Chronic glomerulonephritis	460	5.4	288	255	543	1.13
	1380	5.0	325	450	775	0.72
Mean	992	5.2	371	384	755	0.98
XXIX	332	5.8	150	226	376	0.66
R = 0.95	320	5.3	184	171	355	1.07
	354	5.4	181	226	407	0.80
Subacute nephritis	330	5.3	206	184	390	1.12
	340	5.3	221	183	404	1.21
Mean	335	5.4	188	198	386	0.97
XXX	1075	6.3	270	344	614	0.79
R = 0.93	660	5.7	250	216	466	1.16
Subacute nephritis						
Mean	868	6.0	260	280	540	0.97

TABLE I—*Continued.*

CASE	V	H	A	NH_3	TA	R
XXXI	1020	5.1	265	336	601	0.79
R = 0.92	800	5.1	193	248	441	0.78
	600	5.1	145	190	335	0.76
	1200	4.7	330	272	602	1.21
	1025	5.0	266	276	542	0.96
	1240	4.8	485	385	870	1.26
	1155	5.3	260	328	588	0.79
	1005	5.3	227	235	462	0.97
	920	5.3	180	230	410	0.78
	740	5.3	193	184	377	1.05
	850	5.4	195	173	368	1.13
	645	5.1	193	163	356	1.18
	700	5.1	214	151	365	1.42
Cardiorenal disease, chronic glo-	600	5.3	110	158	268	0.70
merulonephritis	1100	5.1	174	280	454	0.62
	600	5.0	160	159	319	1.00
	650	5.3	174	220	394	0.79
	500	5.3	133	143	276	0.93
	775	5.4	204	247	451	0.83
	800	5.3	176	220	396	0.80
	540	5.3	130	177	307	0.73
	700	5.4	197	228	425	0.86
	480	5.6	138	143	281	0.96
	800	5.3	232	214	446	1.08
	730	5.0	170	152	322	1.12
	1400	5.2	217	256	473	0.85
	1300	5.2	215	286	501	0.75
	800	5.0	218	225	443	0.97
Mean............................	953	5.2	207	224	431	0.93
XXXII	1225	7.2	90	125	215	0.42
R = 0.83	850	6.0	145	150	295	0.97
	1180	5.4	195	205	400	0.95
Chronic nephritis	1000	5.8	155	218	373	0.72
	1200	5.4	264	285	549	0.93
Mean............................	1091	5.9	166	201	367	0.80

TABLE I—*Continued.*

CASE	V	H	A	NH_3	TA	R
XXXIII	1500	4.8	370	470	840	0.79
R = 0.82	1500	5.0	304	500	804	0.61
	1380	4.8	400	490	890	0.82
	1475	5.0	304	400	704	0.76
	900	4.8	450	450	900	1.00
Cardiorenal disease	930	4.8	545	630	1175	0.87
	990	4.8	380	450	830	0.84
	1380	5.0	382	490	872	0.78
	1650	4.8	536	590	1126	0.91
Mean	1301	4.9	408	497	905	0.82
XXXIV	1200	5.1	262	320	582	0.82
R = 0.80	690	5.1	180	200	380	0.90
	780	5.1	232	258	490	0.90
	2850	5.3	500	570	1070	0.88
	2500	5.8	340	580	920	0.59
	1700	5.8	240	360	600	0.67
Arteriosclerosis, chronic nephritis	1340	5.3	415	345	760	1.20
	1120	5.0	322	380	702	0.85
	1920	5.1	396	590	986	0.67
	1800	5.4	356	440	796	0.81
	1840	5.8	336	425	761	0.79
Mean	1613	5.3	325	406	731	0.83
XXXV	1120	5.9	129	258	387	0.50
R = 0.77	1640	6.2	109	250	359	0.44
	940	6.0	235	292	527	0.81
Chronic nephritis	1025	5.7	354	350	704	1.01
	920	5.5	385	408	793	0.94
	750	5.3	420	486	906	0.81
	1220	5.3	412	600	1012	0.69
Mean	1088	5.7	292	378	670	0.74
XXXVI	1100	5.3	343	785	1128	0.44
R = 0.76	950	5.3	550	540	1090	1.02
Acute nephritis	700	5.3	472	465	937	1.02
Mean	916	5.3	455	596	1051	0.83

TABLE I—*Continued.*

CASE	V	H	A	NH_3	TA	R
XXXVII	1470	5.6	141	216	357	0.65
R = 0.75	1140	5.0	280	340	620	0.83
Cardiorenal disease	1540	4.8	216	324	540	0.67
	1440	4.7	180	210	390	0.86
	1200	4.8	147	190	337	0.77
Mean	1358	5.0	193	256	449	0.76
XXXVIII	1600	6.0	160	178	338	0.90
R = 0.73	1940	6.1	188	225	413	0.84
Arteriosclerosis (of kidneys)	1450	6.1	163	250	413	0.65
	1700	5.7	195	272	467	0.72
	2500	5.8	230	356	586	0.65
Mean	1838	5.9	187	256	443	0.75
XXXIX	825	6.0	161	370	531	0.44
R = 0.70	2520	5.6	176	360	536	0.49
	4000	7.2	240	350	590	0.69
	2300	7.4	0	220	220	—
	1800	7.4	0	160	160	—
Arteriosclerosis	1740	7.4	0	143	143	—
	1300	7.0	138	195	333	0.71
	990	5.5	394	210	604	1.88
	1150	5.3	390	230	620	1.70
	700	5.3	264	286	550	0.92
Mean	1733	6.4	176	252	428	0.68
XL	440	5.7	193	238	431	0.81
R = 0.68	495	5.8	200	228	428	0.88
	640	6.0	81	220	301	0.37
	668	6.7	80	204	284	0.39
	537	6.0	183	264	447	0.69
	635	6.7	127	316	443	0.40
	744	5.6	182	325	507	0.56
	619	5.5	182	400	582	0.46
Acute nephritis	338	5.8	184	386	570	0.48
	696	5.7	113	437	550	0.26
	297	5.6	126	347	473	0.36
	1075	5.7	94	278	372	0.34
	338	5.5	144	292	436	0.49
	240	5.7	140	228	368	0.61
	250	5.5	172	253	425	0.68
	240	5.5	195	270	465	0.72
	360	5.7	185	250	435	0.74

TABLE I—*Concluded.*

CASE	V	H	A	NH_3	TA	R
XL—*Continued*	360	5.7	179	230	409	0.78
R = 0.68	325	5.5	202	244	446	0.83
	404	5.8	280	302	582	0.93
	685	6.9	202	293	495	0.69
Acute nephritis	675	5.5	385	385	770	1.00
	645	5.6	279	307	586	0.91
	1210	5.2	600	465	1065	1.29
	910	5.3	495	505	1000	0.98
	700	5.2	383	555	938	0.69
Mean	482	5.7	215	316	531	0.63
XLI	1000	6.8	160	288	448	0.56
R = 0.67	750	7.0	106	263	369	0.40
	520	5.4	165	125	290	1.32
Chronic nephritis, uremia	915	5.4	300	335	635	0.90
	980	5.8	135	264	399	0.51
	610	5.7	183	294	477	0.62
Mean	796	6.0	175	261	436	0.72
XLII	1225	5.0	441	680	1121	0.65
R = 0.61	925	4.8	337	508	845	0.66
Chronic glomerulonephritis	1120	5.0	297	570	867	0.52
Mean	1090	4.9	358	586	944	0.61
XLIII	1600	5.3	304	540	844	0.56
R = 0.61	700	5.7	240	385	625	0.62
	800	5.5	212	366	578	0.58
	1700	5.4	340	630	970	0.54
Chronic nephritis	825	5.3	125	220	345	0.57
	1575	5.7	250	420	670	0.60
	1140	5.3	294	356	650	0.83
	1000	5.4	240	356	596	0.67
	760	6.0	104	174	278	0.60
Mean	1122	5.5	234	383	617	0.62
XLIV	1030	5.1	510	620	1130	0.82
R = 0.58	560	5.1	230	310	540	0.74
	660	5.3	306	645	951	0.48
Chronic glomerulonephritis	350	5.3	210	463	673	0.45
	440	5.3	264	530	794	0.50
	840	5.3	460	850	1310	0.54
	600	5.1	232	420	652	0.55
Mean	640	5.2	316	548	864	0.58

1; while, on the other hand, the majority of the daily values of R in the cases of Group 2 are always low. This separation of our cases of nephritis into two discrete groups, though it need not necessarily depend upon the difference between two quite separate and distinct nephropathies, must have a substantial cause, and, accordingly, it will be necessary to consider the characteristics of the two groups of cases. We have, accordingly, undertaken a careful analysis of our data. A large part of this we shall not present here, for it seems better to postpone it as material for a technical paper on the use of statistical methods in the study of renal functions. In order more clearly to show the completeness of the separation of our cases into two groups according to the values of R, it will suffice to consider all cases in which we have determinations upon at least five days, both of nephritics and of normal individuals, and to calculate the mean values of R together with the "probable errors" of this quantity. Such results give the ranges within which the values of R for the cases in question probably fall. In other words, these ranges are such that there is an even chance, if the cases could be preserved in the condition which characterized the period of observation, and studied for a very long period, that the mean values of R for this period would fall within the range of the probable error around the mean values of our actual observations. These values are tabulated below.

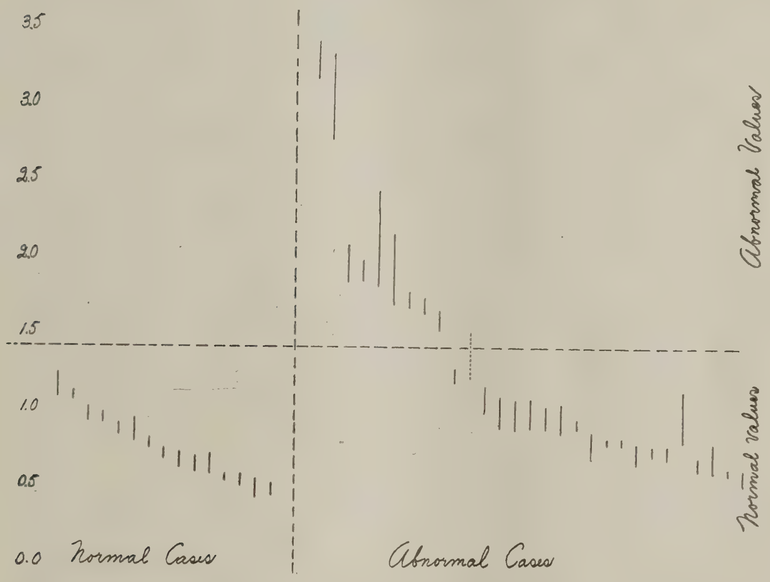
TABLE II.

CASE	R	CASE	R
II	3.33 ± 0.12	XXVIII	0.98 ± 0.06
III	3.09 ± 0.27	XXIX	0.97 ± 0.08
VI	1.98 ± 0.12	XXXI	0.93 ± 0.03
VIII	1.93 ± 0.06	XXXII	0.80 ± 0.08
IX	2.15 ± 0.30	XXXIII	0.82 ± 0.02
X	1.95 ± 0.23	XXXIV	0.83 ± 0.03
XI	1.75 ± 0.04	XXXV	0.74 ± 0.06
XII	1.71 ± 0.04	XXXVII	0.76 ± 0.03
XIII	1.62 ± 0.06	XXXVIII	0.75 ± 0.04
XVII	1.25 ± 0.04	XXXIX	0.98 ± 0.16
XVIII	1.39 ± 0.17	XL	0.67 ± 0.04
XXI	1.10 ± 0.08	XLI	0.72 ± 0.10
XXII	1.01 ± 0.11	XLIII	0.62 ± 0.02
XXIV	1.01 ± 0.10	XLIV	0.58 ± 0.04
XXVII	1.01 ± 0.09		

These values are also expressed graphically in the accompanying diagram, in which each line represents the range of a particular case.

It needs no discussion to show the discontinuity of the abnormally high values of R in nephritis with those which, though often a little elevated, on the whole coincide with the normal values, and are certainly continuous with them.

For convenience the means of all the values of, I, the cases in which R is high (R > 1.6); II, the cases in which R is of medium



value (1.3 > R > 0.8); III, the cases in which R is low (R < 0.8), and, finally, for comparison, the normal values, are all assembled in the following table. To these mean values the probable errors are appended.

TABLE III.

TYPE	V	H	A	NH_3	TA	R
High.....	1650±169	5.2±0.05	318±17	165±14	483±29	2.08±0.14
Medium..	1086±114	5.2±0.08	287±22	276±20	563±42	1.03±0.03
Low.....	1187± 93	5.6±0.10	244±18	341±25	585±42	0.72±0.02
Normal..	1206± 61	5.9±0.04	275±10	368±14	643±17	0.78±0.04

These tables contain the more important results of the present research. They confirm previous observations upon the heightened acidity of the urine in nephritis, a condition which is accompanied, and probably caused by a diminished elimination of acid ($A + NH_3$). Both of these conditions are most marked in the cases in which R is high or at least above the normal average, and far less considerable in those cases which are marked by low values of R. The diminution of acid excretion, however, is not equally conspicuous in the two moieties A and NH_3; for, while the former remains on the whole nearly normal, the value of NH_3 is greatly diminished (necessarily under the circumstances) when R has an abnormally high value, and even when R is only of high normal range.

In the "high" cases the value of A is in fact a little above normal. This, however, seems to be due to the increased intensity of acidity in a phosphate mixture of about the same concentration as in the normal cases. In these cases the ammonia excretion is very greatly diminished, so that on the average it amounts to less than half the normal quantity. This diminution, therefore, is alone responsible for interference with the regulation of the alkalinity of the blood, a disturbance which has been demonstrated by the studies reported in the following communication. Thus these high cases reveal the hitherto unknown phenomenon of a condition of acidosis accompanied by a constant diminution of the urinary ammonia.[4] In these cases the acidity is very high, and the volume is notably above the normal value.

The "medium" cases appear to be of a different kind. They are also, to be sure, characterized by very high acidity, and the total elimination of acid is, though in a less degree, diminished. But the excretion of ammonia is relatively very much less diminished. The volume, instead of being increased, is diminished. The low cases seem to resemble the medium cases, but to be marked by less intense disturbances. The acidity is moderately intense, the total excretion of acid is but little below normal, as a result of a small diminution of A and of an apparently normal excretion of ammonia. But the urinary volume is again low.

[4] Henderson and Palmer: Proc. Am. Soc. Biol. Chemists, this *Journal*, xiv, p. xxv, 1913.

In view of the necessity of very large numbers of observations in order that averages such as are assembled in the above table may be accepted with complete confidence, we do not feel disposed to regard all these fluctuations as significant, but, at least, of the great and constant diminution of ammonia in the high cases there can be no doubt, and as has been shown, there is also good ground for regarding these cases as really different from the others. On the other hand, there is no reason to suppose that the medium and low cases are different from each other save in degree, while one is justified in assuming that however great their difference from the high cases there can hardly be a differentiation into two mutually exclusive types of nephritis.

The nature of this question is such that pathological and even clinical observations may aid in reaching a conclusion. In Table I with each case we have given the clinical diagnosis as it exists in the hospital records, and in no case has any influence on the character of the diagnosis been made by the authors. These diagnoses were made by one of the four visiting physicians to the hospital under whose charge the patient chanced to be.

TABLE IV.

Group 1. R greater than 1.43 Cases I to XVI		Group 2. R less than 1.43 Cases XVII to XLIV	
Chronic glomerulonephritis	9	Chronic glomerulonephritis	8
Arteriosclerosis	2	Arteriosclerotic nephritis; Chronic interstitial nephritis	5
Chronic nephritis	2	Chronic nephritis	5
Acute nephritis	2	Cardiorenal disease	4
Syphilitic nephritis	1	Subacute nephritis	4

The cases are collected in Table IV with reference to these diagnoses and are divided into two groups, one with ratios greater than, a second with ratios less than 1.43.

The clinical course and other data, as well as a detailed discussion of the significance of the high and low ratios in reference to the clinical and anatomical diagnosis and prognosis in individual cases will appear in a subsequent paper. Here we wish merely to call attention to the predominance of chronic glomerulonephritis in the group with high ratios and the large proportion of cases

with degenerative nephritis in the group with low ratios. Of the sixteen high ratio cases nine were definitely diagnosed as chronic glomerulonephritis. It is fair to say that the two cases with the diagnosis of acute nephritis might have been merely acute exacerbations of a slowly progressing nephritis. The two cases called chronic nephritis were apparently borderline cases in which a definite diagnosis could not be made. Syphilitic nephritis obviously was a diagnosis of convenience and may properly be considered under the head of chronic glomerulonephritis. Practically all of the cases in Group 1 were more or less uremic.

In the second group the cases seem more scattered among the various diagnoses, but if the cases which have been catalogued as arteriosclerotic nephritis, chronic interstitial nephritis, chronic

TABLE V.

CASE	R	CLINICAL DIAGNOSIS	ANATOMICAL DIAGNOSIS
VII.....	1.93	Chronic glomerulonephritis	Arteriosclerotic nephritis.
XI*....	1.72	Chronic glomerulonephritis	Chronic interstitial nephritis.
XLI....	0.67	Chronic nephritis	Arteriosclerotic nephritis.
XLIII....	0.61	Chronic nephritis	Arteriosclerotic nephritis.

* The necropsy on this case was performed at the Peter Bent Brigham Hospital, to which I am indebted for being able to include this case among those with anatomical diagnoses.

nephritis, and cardiorenal disease are taken together, exactly half the cases are included. This grouping seems justifiable because the degenerative type of nephritis in most instances is indicated. In contrast to the cases with high ratios only 8, or 22 per cent, have been called chronic glomerulonephritis. None of the cases diagnosed as acute, subacute, or chronic glomerulonephritis with low ratios, either clinically or by the various functional tests used, showed marked renal insufficiency. On the other hand, several among the cases of degenerative nephritis were uremic and a few died in typical uremia. Unfortunately only four of the forty-four cases reported were autopsied.

The comparison between the clinical and anatomical diagnosis in these cases appears in Table V.

Taking all these facts into account, we feel justified in drawing the conclusion that our cases of nephritis divide themselves into two groups possessing the following characteristics:

I. Cases in which the volume of urine is abnormally great, its acidity abnormally intense, and the total acid excretion much diminished (signs of a condition of acidosis which may be of renal origin). This diminished acid excretion is due exclusively to a never failing deficit in the urinary ammonia; for the value of A is, taking account of the intensity of acidity, precisely normal.

II. Cases in which the mean urinary volume appears to be not far from normal, the acidity high and often very high, the total acid excretion often low, but not infrequently normal. The variation in this quantity is once more due to fluctuations in the urinary ammonia. These cases suggest the idea that they involve varying degrees of acidosis which are generally much milder than in the cases of Group 1.

Group 1 appears to consist of an uncommonly sharply defined group of cases which, functionally at least, are of one type. Group 2 may well consist of either one or more classes of disturbance of renal function, including perhaps mild forms of the condition represented in Group 1.

ON THE RETENTION OF ALKALI IN NEPHRITIS.

By WALTER W. PALMER[1] and LAWRENCE J. HENDERSON.

(*From the Wards and Chemical Laboratory of the Massachusetts General Hospital, Boston.*)

(Received for publication, February 27, 1915.)

In the course of the investigations reported in the preceding communication, evidence has steadily accumulated in support of the view that nephritis commonly involves a state of acidosis. At any rate, the more extreme disturbances of the acid excretory function which have come to light hardly seem open to any other interpretation. We have accordingly thought it well to observe the action of sodium bicarbonate, when fed, upon the composition of the urine in these cases, just as we have previously done in a variety of conditions taken at random.

The nature of the observations will be sufficiently clear from the tables of data presented below, which represent fair samples of cases in which ingested alkali has been retained.

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TABLE I.

Observations on single specimens of urine throughout the twenty-four hours.

Case 1. R = 1.63 | *Case 2. R = 2.07* | *Case 3. R = 1.05*

DATE	TIME	V	H	N_aHCO_3	DATE	TIME	V	H	N_aHCO_3	DATE	TIME	V	H	N_aHCO_3
Jan. 1914				*gm.*	Jan. 1914				*gm.*	Jan. 1914				*gm.*
14–15	9.00 a.m.	134	5.1	10	2–3	9.00 a.m.	210	5.1		21–22	8.30 a.m.	160	5.0	
	11.30 a.m.	86	5.1			10.00 a.m.	175	5.1			9.50 a.m.	100	5.1	
	1.00 p.m.	46	5.3			8.00 p.m.	105	5.0			12.50 p.m.	230	4.8	
	1.45 p.m.	30	5.3			10.55 p.m.	130	5.0			5.15 p.m.	235	5.0	
	4.00 p.m.	94	5.1			12.30 p.m.	230	5.0			1.50 p.m.	300	5.0	
	6.40 p.m.	129	5.1			3.00 a.m.	160	5.0		22–23	8.20 p.m.	300	5.1	10 gm. at 10 a.m.
15–16	7.00 a.m.	410	5.3			4.30 a.m.	220	5.0			12.00 n.	150	5.1	
	8.00 a.m.	120	5.1		3–4	8.45 a.m.	175	5.1	5		5.00 p.m.	230	5.1	
	11.10 a.m.	110	5.1	10		12.30 p.m.	195	5.1		23–24	12.25 a.m.	260	5.3	
	12.30 p.m.	30	5.3			3.35 p.m.	165	5.3	5		5.30 a.m.	300	5.4	5 gm. at 10 a.m.
	2.10 p.m.	70	5.1	10		6.45 p.m.	115	5.4			2.30 p.m.	310	5.0	5
	—	85	5.3			8.00 p.m.	150	5.8			4.45 p.m.	100	5.1	
	—	65	5.3	10 gm. at 6 p.m.		1.00 a.m.	160	5.9			5.40 p.m.	50	5.3	5
	—	95	5.3		4–5	1.30 p.m.	135	5.9	5		10.30 p.m.	240	5.3	
	7.00 a.m.	190	7.0			6.45 p.m.	165	6.1			3.30 a.m.	210	5.5	
						12.50 a.m.	135	5.7	5		5.30 a.m.	245	5.3	
						2.00 a.m.	270	6.0		24–25	8.30 a.m.	75	5.1	10 gm. at 10 a.m.
						6.30 a.m.	175	6.3			1.00 p.m.	260	5.1	10
											4.00 p.m.	35	5.3	
											9.20 p.m.	225	5.3	10
											12.45 a.m.	260	6.1	
											3.40 a.m.	150	6.3	

TABLE II.

Observations on daily amounts of urine during ingestion of sodium bicarbonate.

V	H	A	NH_3	TA	R	$NaHCO_3$
						gm.
1380	5.0	382	490	872	0.78	
1650	4.8	536	590	1126	0.91	
845	5.0	320	374	694	0.86	5
990	5.0	419	344	763	1.22	10
2070	7.2	−8	122	114	—	40
700	5.0	282	148	430	1.90	
810	4.8	284	190	474	1.50	
700	5.0	238	200	438	1.19	
750	5.0	292	214	506	1.36	10
929	5.3	306	242	548	1.26	30
765	5.5	180	84	264	2.14	
1055	7.0	52	85	137	0.61	5
750	5.0	490	425	915	1.15	
630	5.1	325	245	571	1.32	
1725	5.1	600	680	1280	0.88	10
1200	5.3	358	428	786	0.89	10
1400	5.3	475	490	965	0.96	15
1240	5.1	351	385	736	0.91	15
1140	5.1	344	392	736	0.88	20
1150	5.1	356	65	421	5.48	20
1100	5.1	320	210	530	1.53	20
950	7.0	100	75	175	1.33	30

In every case of nephritis in which the condition of diminished ammonia excretion (high R of the preceding paper) was detected, there has been a real retention of alkali. This is also commonly the case with individuals representing other types of nephritis (low R), but not invariably so.

Table III contains in summary form our principal systematic observations upon the amount of alkali which needs to be fed in order to produce alkalinity of the urine.

In spite of irregularities, there can, we believe, be no doubt that these results point to an invariable association of acidosis with the one type of nephritis and a frequent association with other types. This investigation, therefore, strengthens the view that it is possible for a condition of acidosis to be accom-

TABLE III.

Amount of alkali necessary before urine became alkaline.

CASE	R	$NaHCO_3$
		gm.
1	1.05	110
2	0.92	65
3	0.82	55
4	1.63	45
5	1.16	40
6	1.08	35
7	2.07	20
8	2.60	15
9	1.89	15
10	1.46	15
11	0.51	5

panied by diminished excretion of ammonia, as well as by heightened excretion.

It may be pointed out that in several instances in these experiments the excretion of ammonia seems to be depressed by the ingestion of alkali before the hydrogen ion concentration yields to the treatment.

THE ESTIMATION OF NON-PROTEIN NITROGEN IN BLOOD.

By ISIDOR GREENWALD.

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(Received for publication, March 29, 1915.)

Of the methods proposed for the estimation of non-protein nitrogen in blood, only that proposed by Folin and Denis[1] has come into general use. The simplicity of this method has commended it to all workers, particularly to those called upon to make a large number of determinations. Nevertheless, no critical study of the method has appeared. Very recently Bock and Benedict,[2] in an examination of part of the technique employed, pointed out certain sources of error and suggested certain modifications for the purpose of increasing the accuracy of the method. The distillation method of Bock and Benedict has recently been employed in this laboratory. It is more convenient and more accurate than the aeration method of Folin and Denis. Duplicate determinations show better agreement and the time required is much less.

The method employed by Folin and Denis for the separation of the protein and non-protein nitrogenous constituents of the blood is open to serious objection. Methyl alcohol is, upon *a priori* considerations, not a good solvent for the extraction of substances, such as some amino-acids, that are comparatively insoluble therein. Indeed, Folin and Denis state that creatine, asparagine, and tyrosine added to blood could not be quantitatively recovered. Moreover, methyl alcohol extracts a varying amount of lipoid nitrogen from blood. It is true that this is non-protein, but it certainly does not belong in the same category as urea and amino-acid nitrogen. The amount, though small, is appreciable. We have evaporated, *in vacuo*, the filtrates obtained

[1] O. Folin and W. Denis: this *Journal*, xi, p. 527, 1912.

[2] J. C. Bock and S. R. Benedict: *ibid*, xx, p. 47, 1915.

in the Folin-Denis procedure and have precipitated the lipoids with hydrochloric acid and chloroform.[3] After washing the precipitate with dilute hydrochloric acid saturated with chloroform, the nitrogen was estimated. The values obtained were, for human serum 2.67 and 2.53 mg., for beef blood 3.12 and 4.28 mg., and for sheep blood 2.82 mg. of nitrogen per 100 cc. of blood.

In view of these sources of error in the method and the undoubted usefulness of estimations of non-protein nitrogen in blood, it seemed desirable to attempt to eliminate them. In the search for a protein precipitant, in addition to complete precipitation of the proteins, three properties were desired: 1. The precipitant should be used in aqueous solution, so as to make more probable the complete extraction of all non-protein, water-soluble substances and also to ensure complete precipitation of the lipoids with the proteins. 2. It should not redissolve protein when present in excess, so as to permit of considerable variation in the proportions of blood-protein and precipitant. 3. It should not interfere with the subsequent digestion or distillation. Most of the usual protein precipitants fail to meet one or more of these conditions. The good results which the author[4] has obtained in the extraction of phosphates from blood by means of a mixture of hydrochloric and picric acids[5] suggested the use of a similar solution.

After a number of experiments with several protein precipitants, it appeared that trichloracetic acid closely approximated the ideal precipitant. It is very soluble in water, is readily volatile, does not dissolve protein when present in great excess, is readily obtained free from nitrogen, and at a comparatively low cost. A few preliminary experiments showed that the proteins of blood are completely precipitated by the addition of nine volumes of a 2 per cent solution. As a routine precipitant, nine volumes of a 2.5 per cent solution were adopted. The action of this solution upon sheep blood was compared with that of a 22 per cent solution. In

[3] W. Koch and H. S. Woods: *ibid.*, i, p. 206, 1905–06.

[4] I. Greenwald: *ibid.*, xiv, p. 369, 1913.

[5] Folin, Lewis and Benedict, and Chapin and Powick have also used picric acid for precipitating proteins and lipoids in the estimation of creatinine, creatine, glucose, and phosphates. (Folin: this *Journal*, xvii, p. 475, 1914. R. C. Lewis and S. R. Benedict: *ibid.*, xx, p. 61, 1915. R. M. Chapin and W. C. Powick: *ibid.*, xx, p. 97, 1915.)

the filtrates there were found, respectively, 36.3 and 36.4 mg. of nitrogen per 100 cc. of blood. Evidently a solution of nine times the concentration ordinarily used did not dissolve appreciable amounts of protein.

The filtrates obtained are colorless, though not always absolutely clear. The slight turbidity can readily be removed by shaking with a little kaolin. The filtrate from the kaolin is absolutely clear and colorless. The liquid remains clear after the addition of picric acid or of potassium-mercuric iodide, even upon standing for twenty-four hours, and gives only the slightest turbidity with phosphotungstic acid.

The precipitates obtained upon the addition of phosphotungstic acid to filtrates corresponding to 46 cc. of sheep blood, to 20 and 25 cc. of human blood, and to 50 cc. of beef blood were used for the determination of nitrogen. The amounts obtained were no larger than in the blanks. In another experiment, 3 liters of filtrate, corresponding to 300 cc. of sheep blood, yielded 0.323 mg. of nitrogen, or 0.108 mg. per 100 cc. of blood.

The absence of protein in these filtrates was demonstrated in other ways.

Large volumes were almost neutralized with sodium hydroxide and evaporated under diminished pressure, with an outside temperature not exceeding 40° to a volume approximately equal to that of the original blood.[6] Portions of the turbid liquid were treated with hydrochloric acid and potassium-mercuric iodide. After standing for from twenty-four to forty-eight hours, the precipitate was centrifugated out, washed with water containing a little of the precipitants, and the nitrogen content estimated. Even without previous treatment of the original filtrates with kaolin, the amount of nitrogen found, per 100 cc. of blood, was, in beef blood, 0.151 and 0.346 mg., in sheep blood 1.56 and less than 1.23 mg., and in human blood 0.21 mg. After treatment with kaolin, 970 cc. of the filtrate from beef blood yielded only 0.067 mg. of nitrogen when treated in this way, or 0.069 mg. per 100 cc. of blood.

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[6] We have, in one experiment, tried evaporation of the neutralized blood filtrate on the water bath. It was found to be strongly alkaline.

$$(2CCl_3COONa + H_2O = Na_2CO_3 + 2CHCl_3 + CO_2)$$

This reaction does not occur when the evaporation takes place at a low temperature. We have always done this in order to avoid this reaction and also the possible decomposition of any protein that might be present, although all our results indicate that there is no protein in these filtrates.

In other experiments large volumes of the trichloracetic acid filtrates were neutralized and evaporated as already described. To these were then added nine volumes of methyl or ethyl alcohol. The precipitate was allowed to settle for at least twenty-four hours and then kept in fresh alcohol for another day. It was then washed with alcohol and dried. Upon treating with water, almost all dissolved. The insoluble material was used for nitrogen estimations. There were obtained, respectively, 0.353 and 0.037 mg. per 100 cc. of sheep and beef blood. The solution of the material precipitated by alcohol did not give the biuret reaction, nor any precipitate with picric acid or potassium-mercuric iodide, and only a very slight precipitate, if any, with phosphotungstic acid, even after standing twenty-four hours. The nitrogen in a particularly heavy precipitate was determined and found to be 0.16 mg. per 100 cc. of blood. Estimations of the total nitrogen and of amino-nitrogen (by Van Slyke's method) in the solution of the alcohol precipitate showed that from 25 to 50 per cent of the nitrogen was present as amino-nitrogen. This was not increased by boiling with hydrochloric acid.

From the above experiments it seems that there is no appreciable amount of protein in the filtrate from trichloracetic acid and kaolin.

That trichloracetic does not split off nitrogen from the proteins of blood is indicated by the following experiment.

Blood was dialyzed against running water for six days. It was then treated with trichloracetic acid and kaolin in the usual manner. Nitrogen was estimated in 50 cc. portions of the filtrate and in the same volume of trichloracetic acid solution, after treatment with kaolin. The same amount was found in both; *viz.*, 0.044 mg. This includes the nitrogen of the Kjeldahl reagents.

The amount of nitrogen extracted from blood by means of trichloracetic acid was compared with that obtained by treatment of another sample of the same blood with hydrochloric acid and mercuric chloride. The amounts obtained were, respectively, 34.1 and 33.7 mg. of nitrogen per 100 cc. of blood.

It remains to discuss the efficacy of the method in the recovery of added amino-acids and to compare the results with those obtained by the Folin method. It was not thought necessary or advisable to make a large number of tests with individual amino-acids but to use a fairly typical mixture.

Casein was hydrolyzed with boiling 25 per cent hydrochloric acid for forty hours. Most of the hydrochloric acid was distilled off under diminished pressure, the liquid was neutralized with sodium hydroxide and

filtered. The filtrate was treated with animal charcoal and filtered. After most of the tyrosine had crystallized out the amino-nitrogen of the final filtrate, determined by Van Slyke's method, was 83 per cent of the total nitrogen. Measured portions of this liquid were mixed with known volumes of blood. Estimations of the non-protein nitrogen in the untreated blood and in the blood and amino-acid mixture were then made. It was found, in every case, that the trichloracetic acid filtrate contained all the added nitrogen, whereas the methyl alcohol filtrates contained little over half. Correspondingly, the nitrogen in the trichloracetic acid filtrates from untreated blood was higher than in the methyl alcohol filtrates. Previous laking of the erythrocytes did not appear to affect the results with trichloracetic acid.

TABLE I.

Recovery of the nitrogen of urea and of amino-acids added to blood.
Mg. per 100 cc. of blood.

MATERIAL	METHYL ALCOHOL		TRICHLORACETIC ACID	
	Found:	Calculated:	Found:	Calculated:
Sheep blood	25.8		36.3	
" " and urea	79.6	80.4	88.9	86.8
" " " amino-acids	43.7	54.4	64.5	64.4
" " " " "	61.1	82.0	90.2	91.5
Pig blood	16.2		31.5	
" " and urea	66.7	71.3	89.1	87.8
" " " amino-acids	33.5	45.3	59.5	59.8
Beef blood	14.6		26.2	
" " and amino-acids	33.5	43.8	56.9	54.8
" " " " "	54.4	73.0	84.6	83.3

In Table II are presented the results of a number of determinations by the two methods. According to the proposed modification, the normal amount of non-protein nitrogen in human blood is about 30 mg. per 100 cc. instead of about 22 mg. In nephritic blood, both methods give higher results, but the difference between the two is not proportionately increased. This is to be expected, as the increase in the non-protein nitrogen of the blood in nephritis is largely due to a retention of urea, which is quantitatively extracted by either method.

In this work the blood has regularly been diluted to ten times

its original volume with the trichloracetic acid solution. The determinations have been made upon measured portions of the filtrate, without washing the precipitate. The error due to the volume of the precipitate appears to be negligible.

Among minor advantages of the use of trichloracetic acid instead of methyl alcohol is the saving in time. The blood and

TABLE II.

Nitrogen extracted by methyl alcohol and by trichloracetic acid from blood. Mg. per 100 cc. of blood or serum.

BLOOD			SERUM		
Diagnosis	Methyl alcohol	Trichlor-acetic acid	Diagnosis	Methyl alcohol	Trichlor-acetic acid
Normal	21.0	27.9	Normal	21.1	29.4
Influenza, pneumonia	35.9	50.5	"	23.7	28.9
Polycythemia	26.2	42.4	"	21.2	31.0
Chronic bronchitis	31.9	43.5	"	22.0	31.7
Chronic endocarditis, rheumatism	31.6	42.7	"	21.2	26.3
			Chronic endocarditis	34.6	47.2
Chronic nephritis	16.9	31.5	Pneumonia	29.8	41.3
" "	18.8	29.5	Chronic nephritis	26.2	33.0
" "	69.9	80.9	" "	23.9	33.8
" "	120.0	137.2	Diabetes, pneumonia, chronic nephritis	66.7	73.4
" "	53.0	72.0	Acute nephritis	35.6	47.2
" "	29.6	35.5			
" "	22.9	32.7			
" "	32.7	44.1			
" "	21.3	31.3			
" "	174.0	195.0			
" "	110.0	133.0			

trichloracetic acid mixture may be filtered within a half hour. If great accuracy is not desired, the treatment with kaolin may be omitted. The results may then be too high, but the error does not exceed 2 mg. per 100 cc. of blood. Because of the absence of fats and similar substances in the solution the oxidation is completed very much sooner than when methyl alcohol is used.

Directions for the determination of non-protein nitrogen in blood.

As the result of our experience we carry out the determination of non-protein nitrogen in the following manner. The blood is diluted to ten times its original volume with 2.5 per cent trichloracetic acid solution. After standing for thirty minutes it is filtered and the filtrate shaken with a small amount of kaolin (about 4 gm. per 100 cc.) and again filtered. (If an error of not more than 2 mg. of nitrogen per 100 cc. of blood may be neglected, the treatment with kaolin may be omitted.) Portions of the filtrate are measured into large Jena test-tubes, as recommended by Folin and Denis;[7] a few small glass beads, 1 cc. of concentrated sulphuric acid, and a few drops of 5 per cent copper sulphate solution are added and the mixture is boiled down until it chars. Approximately 0.3 gram of potassium sulphate is then added and the heating is continued for five minutes after the mixture has become colorless. It is then allowed to cool, is diluted, and, after adding a little powdered pumice stone, distilled as recommended by Bock and Benedict.[8] The subsequent procedure is that of Folin and Denis. It is most convenient to use 10 cc. of filtrate, equivalent to 1 cc. of blood, and to dilute the distillate, after Nesslerizing, to 50 cc. The non-protein nitrogen of the specimen may then lie between 25 and 90 mg. per 100 cc. of blood without impairing the accuracy of the determination.

It is with great pleasure that I acknowledge my indebtedness to Mr. Morris Weiss for his assistance in the experimental work, and to Mr. Kanematsu Sugiura for the estimations of amino-nitrogen.

SUMMARY AND CONCLUSIONS.

The use of methyl alcohol as a precipitant for proteins in the determination of non-protein nitrogen is undesirable for several reasons. Amino-acids added to blood cannot be quantitatively recovered in the filtrate. Moreover, the extract contains about 3 mg. of lipoid nitrogen per 100 cc. of blood. This, though non-protein, scarcely belongs in the same fraction as urea and

[7] Folin and Denis: *loc. cit.*

[8] Bock and Benedict: *loc. cit.*

amino-acid nitrogen. If a 2.5 per cent solution of trichloracetic acid be substituted for the methyl alcohol, the lipoids are completely precipitated with the proteins, and added amino-acids may be quantitatively recovered in the filtrate. This may contain a trace of protein, which can be removed by shaking with a little kaolin. The final filtrate appears to be free from protein and to contain all the water-soluble, non-protein constituents of the blood. The normal amount of non-protein nitrogen is thus found to be about 30 mg. per 100 cc. of human blood. It seems, therefore, that not only are added amino-acids not completely recovered by extraction with methyl alcohol, but that a considerable amount of non-protein nitrogen normally present in blood, the exact nature of which is yet to be ascertained, also fails of extraction.

A NOTE ON THE DETERMINATION OF NITROGEN BY THE KJELDAHL-FOLIN-FARMER METHOD.

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The use of the Kjeldahl-Folin-Farmer[1] method for the determination of the total nitrogen content of biological fluids has proved of such enormous value to chemists and physiologists that the authors were of the opinion that the method could be applied in pure organic chemistry to the determination of the percentage of nitrogen in organic compounds. Cases frequently arise in which the amount of an organic compound, produced in synthesis or by analysis, is too small for a determination of its percentage of nitrogen to be carried out by the Dumas method or the ordinary Kjeldahl-Gunning process. The identification of minute amounts of organic bases, the determination of the composition of rare alkaloids, where it is necessary to conserve the supply of material, are cases to which the determination of nitrogen by the two above mentioned methods could not be applied with success. Each of them requires at least 0.2 gram of substance for determinations in duplicate, whereas the small amount of material required in the micro Kjeldahl process of Folin and Farmer seemed to hold out a promise of success and open up fields of investigation which had previously been closed.[2]

The authors, however, were not aware at the time of the limits of accuracy of this method, and in order to ascertain this point, they made a series of determinations of the percentage of nitrogen in urea. Since this experimental work a paper by Bock and Benedict[3] has appeared in which the Folin-Farmer modification of the Kjeldahl process is adversely criticized. The number of experiments performed and the invariable discrepancy of the

[1] O. Folin and C. J. Farmer: this *Journal*, xi, p. 493, 1912.
[2] E. C. Grey: *Tr. Chem. Soc.*, cv, p. 2204, 1914.
[3] J. C. Bock and S. R. Benedict: this *Journal*, xx, p. 47, 1915.

results show quite clearly the limitations of the method. Our own results bring out the same point, but not to so great an extent; indeed, the errors of experiment are found to be compensated provided a sufficient number of determinations be performed. Such a number of experiments, however, from our point of view was not desirable; for the fact that a greater number than two has to be performed in order to obtain a result in harmony with theory destroys one's confidence in the method, and consequently we abandoned the process as a means of determining the percentage of nitrogen in minute amounts of organic compounds. Bock and Benedict's paper, however, opens the subject in a more general way and hence we think our experimental results will prove of interest to others besides ourselves.

In our experimental procedure we followed the details given by Folin and Farmer, except that our ammonia was aspirated in the $\frac{N}{10}$ HCl placed in a 100 cc. flask and Nesslerized direct without transference to another vessel.

0.02 gm. of urea was dissolved in water and made up to 10 cc., and the nitrogen was determined in 1.0 cc. The colorimeter readings given represent the mean of five consecutive closely agreeing readings.

COLORIMETER READING	N_2 PER LITER	N_2 IN UREA
cm.	*gm.*	*per cent*
2.17	0.922	46.08
2.15	0.930	46.51
2.12	0.943	47.12
2.16	0.926	46.30
Average 2.15	0.930	46.50
Theory..................	0.933	46.60

Thus it will be seen that the average of these four determinations gives a result in close agreement with theory. The errors of the individual determinations are compensated in this particular case, though they are large when reckoned in percentage of nitrogen. A difference of 0.02 cm. in the colorimeter readings means a difference of nearly half a per cent on the percentage of nitrogen, an error too great to be allowed in the calculation of the formula of an organic compound. That errors of this magnitude

are the normal errors of the method can be seen by comparing the results, when expressed as grams of nitrogen per liter, with those given in the original paper of Folin and Farmer. The following figures taken from that paper show the error of the method and the comparison with the Kjeldahl method. The results were expressed in grams of nitrogen per liter of urine.

FOLIN METHOD		KJELDAHL METHOD
gm.		*gm.*
7.9	8.1	8.0
3.7	4.1	3.7
10.5	10.0	10.2
9.4	9.3	9.2
9.3	9.1	9.2

The small amount of divergence of the colorimeter readings in our determinations is, we believe, a normal divergence, and is inherent not only in this particular colorimetric process but in all determinations depending on the use of the colorimeter. The results of one of us with Mr. MacLean[4] on a colorimetric determination of amino-acid α-nitrogen show this same divergence,—about 1 per cent. Small as this is, and confirming the value of the Kjeldahl-Folin-Farmer method for the uses for which it was designed, *viz.*, the determination of total nitrogen in minute amounts in physiological fluids, it is too large to allow of the extension of the method to those problems which we have indicated, and we are reluctantly compelled to abandon it.

[4] V. J. Harding and R. M. MacLean: *ibid.*, xx, p. 217, 1915.

THE DETERMINATION OF THE SULFOFYING POWER OF SOILS.

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All plants require sulfur in greater or lesser amounts for their growth, but while this fact has long been recognized, it is only recently that this element has begun to receive the attention which its importance demands. Improved methods[1] of crop analysis have shown that ordinary farm crops remove considerably larger amounts of sulfur from the soil than had previously been supposed, and the necessity of insuring a proper supply of this element in soluble form, if crops are to continue to receive an adequate supply, becomes, therefore, quite evident.

It is known that sulfur occurs in soils mainly in complex organic compounds, only small amounts of sulfites, sulfates, and sulfides, and other mineral sulfur compounds being present. It is likewise known that plants require sulfur in the form of sulfates, and hence it is evident that the process of the transformation of organic compounds containing sulfur into sulfates is of great importance from the standpoint of the feeding of crops. This transformation or oxidation has been termed "sulfofication" by Lipman[2] in his admirable scheme of nomenclature for bacteriological processes in the soil, and it will be employed in this work as a general term to include the oxidation of organic sulfur compounds, sulfides, and free sulfur with the production of sulfates.

It has been recognized that bacteria are active in the oxidation of sulfur compounds, but their activities in soil in bringing about a production of sulfates have not been studied to any

[1] E. B. Hart and W. H. Peterson: *Research Bulletin 14, Wisconsin Agricultural Experiment Station*, 1905.

[2] J. G. Lipman: *Botan. Gaz.*, li, p. 454, 1911.

extent previously. It is evident, however, that if they are weak and inefficient, sulfate production might be expected to be slow and crops to suffer, even though an abundance of total sulfur was present in the soil.

The present work deals mainly with the questions: Do soils have a sulfofying power? And if so, how may it be determined? A study of other questions is being carried out, and many data are being accumulated which will be published later. The experiments described here are those which have shown that soils *do* have a sulfofying power; that this power is determinable by laboratory methods; and that the physical characteristics of soils and certain methods of soil treatment influence to a considerable extent the ability of a soil to produce sulfates.

HISTORICAL.

Very little work has been done on the problem of sulfur transformation in soils but it has been found that bacteria bring about its oxidation to sulfates, in which form it is assimilable by plants

Degrully[3] applied sulfur to soil, found the greater part of it appearing later as sulfate, and concluded that the increased crop was due to the sulfates formed, or to a stimulating effect of the sulfur on the plants. Demolon[4] found that sulfur was oxidized to sulfate in the soil, and in later experiments[5] he confirmed this observation and concluded that the fertilizing action of sulfur was due partly to the formation of sulfuric acid, which either acts directly as a source of sulfur, or by its action on bases, especially calcium, makes more mineral matter available to plants. Kossovitch[6] has emphasized recently the fact that sulfur passes through a cycle in nature from organic to inorganic form, undergoing oxidation and reduction principally through the activities of microörganisms. Brioux and Guerbet[7] concluded from their investigations that the oxidation

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[3] L. Degrully: *Prog. Agr. Vit.*, lvii, p. 321, 1912; *Chem. Abstr.*, vi, No. 12, p. 1649, 1912.

[4] A. Demolon: *Compt. rend. Acad. d. sc.*, cliv, p. 514, 1912; *Chem. Abstr.*, vi, No. 15, p. 2129, 1912.

[5] Demolon: *Compt. rend. Acad. d. sc.*, clvi, p. 725, 1913; *Chem. Abstr.*, vii, No. 16, p. 2822, 1913.

[6] P. Kossovitch: *Russ. J. Expt. Landw.*, xiv, p. 181, 1913; *Chem. Abstr.*, viii, No. 5, p. 978, 1914.

[7] C. Brioux and M. Guerbet: *Compt. rend. Acad. d. sc.*, clvi, p. 1476, 1913; *Experiment Station Record*, xxx, No. 3, p. 232, 1913.

of sulfur was due to complicated bacterial processes probably involving a number of different kinds of bacteria. They found also that sugar and starch apparently retarded oxidation, while peptone and other nitrogenous substances favored it. Calcium carbonate was shown to accelerate the oxidation, but sterilization entirely prevented it. This would indicate that the oxidation of free sulfur occurs entirely by bacterial means and not by chemical.

From these few experiments which have considered the bacterial phases of the problem of sulfur fertilization, the conclusion has been drawn that the process of sulfofication is brought about entirely by bacterial agency. The data presented in support of this contention are regarded as rather insufficient, however, to prove the point. Certain results obtained in this work tend to show that there may be a small chemical oxidation of sulfur compounds in the soil, but that the major part of the process of sulfofication is brought about by bacterial means.

While it has been shown, therefore, that bacteria are active in the process of sulfur oxidation, no attempt has been made previously to determine the sulfofying power of soils, or their ability to produce sulfates.

EXPERIMENTAL.

Profiting by the experience undergone in developing methods for ammonification, nitrification, and azofication, it was decided to work with fresh soil, attempting by its use to imitate field conditions as closely as possible, and to insure thereby the applicability of the results secured to field soils. The solution method of testing bacterial activities has proved in our experience so much less satisfactory than the soil method that it was not employed here.

Known amounts of different sulfides or of free sulfur were added to 100 gram portions of soil, and the amount of sulfate which formed during a period of incubation was determined. The results obtained in this way indicate whether, in the presence of abundance of sulfur, other conditions being satisfactory, this element may be transformed into sulfates fast enough to keep the plant supplied with the necessary amount.

In all this work sulfates have been determined according to the method devised by the writers, which consists in shaking 100 grams of soil with 200 cc. of water for seven hours in the shak-

ing machine, filtering, precipitating with $BaCl_2$, and estimating in the sulfur photometer.

Series I. Oxidizability of different sulfides in soil.

0.1 gram each of potassium, sodium, and calcium sulfide was added in duplicate to 100 gram quantities of fresh soil in tumblers, the moisture content adjusted to the optimum, and the tumblers covered and incubated for five days at room temperature. At the end of this time the sulfates were determined.

The results given in Table I show large sulfate production from the sulfides added. Sodium sulfide was more readily or quickly oxidized than the other sulfides, and hence it was used in the following series.

TABLE I.

LAB. NO.	ADDITION	S AS SULFATE	S AS SULFATE (AVERAGE)	S AS SULFATE PRODUCED FROM SULFIDE ADDED	S ADDED AS SULFIDE	SULFUR ADDED OXIDIZED
		mg.	*mg.*	*mg.*	*mg.*	*per cent*
1	Nothing	3.40				
2	"	3.43	3.41	—	—	—
3	0.1 gm. K_2S	15.61				
4	" " "	15.61	15.61	12.20	29.09	41.90
5	0.1 gm. Na_2S	9.73				
6	" " "	10.12	9.92	6.51	13.33	48.83
7	0.1 gm. CaS	15.39				
8	" " "	12.88	14.13	10.72	44.44	24.12

Series II. Effect of previous treatment on the oxidizing power of soil.

100 gram quantities of fresh soils from plots under various treatments were weighed out in tumblers, 0.1 gram of sodium sulfide was added to each, the moisture content adjusted to the optimum, and the samples were incubated as before. The sulfates were leached and determined as usual. The results given in Table II show quite distinctly that there may be considerable variation in the sulfate-producing or sulfofying power of soils. There was only a small variation in the amounts of sulfates present as such in the soils, and hence the final differences were

due practically entirely to variations in the sulfofying powers of the soils.

The oxidation of the sulfides was so very rapid in this last series that it occurred to us that perhaps the action was not entirely bacterial in nature, and that there might be some chemical action involved. Hence it was decided to ascertain whether by shaking a sulfide with soil for seven hours there would be any production of sulfates.

TABLE II.

LAB. NO.	PLOT NO.	TREATMENT	S AS SULFATE	S AS SULFATE (AVERAGE)	S AS SULFATE FROM SOILS	S AS SULFATE PRODUCED FROM SULFIDE	S ADDED AS SULFIDE	SULPHUR ADDED OXIDIZED
			mg.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
1	102	2.8 t. peat	9.21					
2	102	" " "	9.66					
3	102	" " "	9.78					
4	102	" " "	9.48	9.53	1.16	8.37	29.09	28.77
5	103	8 t. manure, once in 4 yrs.	12.44					
6	103	" " " " " "	12.82	12.63	2.06	10.57	29.09	36.33
7	104	8 t. clover, once in 4 yrs.	13.32					
8	104	" " " " " "	13.32	13.32	1.66	11.66	29.09	40.08
9	106	2 t. timothy	19.02					
10	106	" "	21.83	20.42	2.26	18.16	29.09	62.42
11	107	Check	20.02					
12	107	"	20.59	20.30	2.66	17.64	29.09	60.63

Series III. Chemical oxidation of sulfides during extraction for analysis.

In order to test this point samples of fresh soil were treated with sodium sulfide as usual, but instead of incubating the samples, the sulfates were determined immediately after the sulfides were added. The results in Table III show that our suspicions were correct, and that there was a chemical oxidation of the sul-

fides in the soil shaken with water for seven hours. The sodium sulfide was affected less than the potassium and calcium sulfides.

It is evident from these results that the percentage oxidation reported in the preceding series did not occur entirely during the incubation. The presumably chemical oxidation undergone by the sulfides when shaken with water should therefore be deducted from the total.

TABLE III.

LAB. NO.	ADDITION	S AS SULFATE	S AS SULFATE (AVERAGE)	S AS SULFATE PRODUCED FROM SULFIDE	S ADDED AS SULFIDE	SULFUR ADDED OXIDIZED
		mg.	*mg.*	*mg.*	*mg.*	*per cent*
1	Nothing	3.58				
2	"	3.58	3.58	—	—	—
3	0.1 gm. K_2S	8.87				
4	" " "	9.25	9.06	5.48	29.09	18.83
5	0.1 gm. Na_2S	5.28				
6	" " "	5.31	5.29	1.71	13.33	12.82
7	0.1 gm. CaS	11.53				
8	" " "	11.89	11.71	8.13	44.44	18.29

Series IV. Chemical oxidation of sulfides mixed with sterile soil and sand when shaken with water for analysis.

In order to eliminate any possibility of bacterial action in the shaking process, this series was planned with sterile soil and sand. 100 gram quantities of these materials, the soil being in an air-dry condition, were weighed out in tumblers and sterilized, additions of sulfides made as before, and the sulfates determined. The results appear in Table IV.

It is apparent that there was very slight oxidation of the sodium sulfide in the sterile soil and none at all in the sand. In the cases of the potassium sulfide and the calcium sulfide, however, there was a considerable oxidation. Evidently a purely chemical oxidation of sulfides may occur when they are shaken with water for seven hours.

An interesting point is here brought out, and one which does not agree with the conclusions from some experiments mentioned in the historical summary. The authors in those cases

concluded that the oxidation of sulfur occurred in the soil by bacterial agency only, but these results indicate that such is not the case. The production of hydrogen sulfide is recognized as a step in the sulfur cycle in nature, but this substance when produced immediately unites with some base to form a sulfide, and hence sulfides such as these used in this series undoubtedly occur in the soil. If there is a purely chemical oxidation of these compounds when shaken with water for seven hours, there is every reason to think that there may be such a change in the soil itself. In other words it seems quite possible from these

TABLE IV.

LAB. NO.	MEDIUM	ADDITION	S AS SULFATE	S AS SULFATE (AVERAGE)	S AS SULFATE PRODUCED FROM SULFIDE	S ADDED AS SULFIDE	SULFUR ADDED OXIDIZED
			mg.	*mg.*	*mg.*	*mg.*	*per cent*
13	Soil	Nothing	4.52				
14	"	"	4.52	4.52	—	—	—
1	"	0.1 gm. Na_2S	4.98				
2	"	" " "	4.54	4.76	0.24	13.33	1.80
3	"	0.1 gm. K_2S	11.34				
4	"	" " "	11.38	11.36	6.84	29.09	23.51
5	"	0.1 gm. CaS	15.25				
6	"	" " "	15.38	15.31	10.79	44.44	24.27
7	Sand	0.1 gm. Na_2S	—				
8	"	" " "	—	—	—	13.33	—
9	"	0.1 gm. K_2S	6.57				
10	"	" " "	7.87	7.22	7.22	29.09	24.81
11	"	0.1 gm. CaS	10.46				
12	"	" " "	10.23	10.34	10.34	44.44	23.26

results that the production of sulfates in the soil is not entirely a bacterial process, at least in certain stages. Further tests will throw additional light on this point.

Series V. Oxidation of sulfides during extraction from different soils.

The next question which arose was whether the extent of oxidation of sulfides shaken with soil and water varies with different soils. Sodium sulfide was chosen as it gave the lowest oxidation in the previous series.

The results in Table V show that the effect of difference in soil is very pronounced. In order to use sulfides as a measure of the sulfofying power of soils it is evident that, as stated above, the amount of sulfate produced by oxidation in the extraction process must be subtracted from the total in order to obtain any idea of the power of the soil itself to form sulfates. Of course, it is realized that this is a somewhat questionable procedure; inasmuch as after incubation in the soil much of the sulfide has already changed to sulfate. Consequently the additional sulfate formed

TABLE V.

LAB. NO.	PLOT NO.	TREATMENT	S AS SULFATE	S AS SULFATE (AVERAGE)	S AS SULFATE FROM SOILS	S AS SULFATE PRODUCED FROM SULFIDE	S ADDED AS SULFIDE	SULFUR ADDED OXIDIZED
			mg.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
1	101	Timothy meadow	Trace					
2	101	" "	Trace	—	—	—	—	—
3	102	2.8 t. peat	Trace					
4	102	" " "	Trace	—	—	—	—	—
5	103	8 t. manure, once in 4 yrs.	3.56					
6	103	" " " " " "	3.76	3.66	2.81	0.85	13.33	6.37
7	104	8 t. clover, once in 4 yrs.	3.99					
8	104	" " " " " "	4.06	4.02	2.11	1.91	13.33	14.32
9	106	2 t. timothy	5.01					
10	106	" " "	5.78	5.39	3.94	1.45	13.33	10.87

during extraction is presumably less in proportion to the amount of oxidizable sulfides present. In fact, where the oxidation in the shaking of the entire 0.1 gram of sulfide added to the soil is as small as is the case with the sodium sulfide, it is quite probable that the change induced by the shaking after incubation would be practically inappreciable. Furthermore it is certain that, even subtracting the amount of sulfates produced in the shaking from the total amount at the end of the incubation there is evidence of a large sulfofying power in the soils, and that the results show the relative sulfofying powers of the different soils.

Series VI. Oxidation of sodium sulfide incubated in different soils.

As the results thus far seemed to indicate that sodium sulfide was the best substance to use as a measure of sulfofication, it was decided to test several soils from various sources for sulfofying power, using this material. Accordingly six soils of as widely

TABLE VI.

SOIL NO.	SOIL SOURCE	WATER IN SOILS	S AS SULFATE	S AS SULFATE (AVERAGE)	S AS SULFATE IN SOILS	S AS SULFATE OXIDIZED BY SHAKING	S AS SULFATE OXIDIZED IN SOILS DURING INCUBATION	SULFUR ADDED OXIDIZED IN SOILS DURING INCUBATION
		per cent	mg.	mg.	mg.	mg.	mg.	per cent
1	Sandy loam graveyard	16.	9.21					
1	"	16.	10.95	10.08	Trace	2.61	7.47	56.03
2	Sandy loam low, poorly drained area	21.	16.91					
2	" " "	21.	17.29	17.10	5.56	3.61	7.93	59.48
3	Heavy, black woodland soil	26.	18.17					
3	" " " "	26.	18.98	18.57	—	*13.13	5.44	40.81
4	Typical sand river bank	11.	4.41					
4	" " " "	11.	4.02	4.21	Trace	Trace	4.21	31.58
5	Wisconsin drift soil untreated	18.	15.55					
5	" " "	18.	15.37	15.46	3.19	2.33	9.94	74.56
6	Wisconsin drift soil manured at rate of 25 t. per acre	15.	12.15					
6	" " " "	15.	13.92	13.03	1.52	1.18	10.33	77.49

* This includes sulfate from soil and that due to oxidation by shaking.

varying character as possible were sampled, weighed out as usual, sodium sulfide was added, the moisture content adjusted to the optimum for all the soils, and the samples were incubated for five days. The amounts of sulfates present as such in the soils were ascertained, and the amounts of the sulfide added oxidized in the different soils in the shaking process were also determined. These two amounts were subtracted from the

total quantity of sulfates produced, and the differences gave the sulfofying powers of the soils. The results are given in Table VI, and they show that soils do have a variable sulfofying power and that this power is dependent on the bacterial conditions mainly, although the chemical character of the soil also exerts some influence. The physical conditions of the soil evidently have an indirect influence because of their effect on the bacteria.

Series VII. Oxidation of free sulfur in different soils.

In connection with the previous series a duplicate was run, the soils being the same, and the conditions of the experiment the same, the only difference being that 0.1 gram of free sulfur was added to the soils instead of the sodium sulfide. The results

TABLE VII.

SOIL NO.	SOIL SOURCE	WATER IN SOILS	S AS SULFATE	S AS SULFATE (AVERAGE)	S AS SULFATE IN SOILS	S AS SULFATE OXIDIZED BY SHAKING	S AS SULFATE OXIDIZED IN SOILS DURING INCUBATION	SULFUR ADDED OXIDIZED IN SOILS DURING INCUBATION
		per cent	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cen*
1	Sandy loam graveyard	16.	6.15					
1	" " "	16.	6.01	6.08	Trace	1.48	4.60	4.60
2	Sandy loam low, poorly drained area	21.	12.43					
2	" " "	21.	11.98	12.20	5.56	1.76	4.88	4.88
3	Heavy, black woodland soil	26.	11.57					
3	" " "	26.	Lost	11.57	—	*9.87	1.70	1.70
4	Typical sand river bank	11.	3.61					
4	" " " "	11.	3.51	3.56	Trace	Trace	3.56	3.56
5	Wisconsin drift soil untreated	18.	10.05					
5	" " " "	18.	10.34	10.19	3.19	1.37	6.63	6.63
6	Wisconsin drift soil manured at rate of 25 t. per acre	15.	12.48					
6	" " " "	15.	13.11	12.79	1.52	0.48	10.79	10.79

* Includes sulfate from soil and that due to oxidation by shaking.

of this series are given in Table VII. The oxidation of the free sulfur by shaking with water was very small, and in some cases practically nothing. The percentage of sulfur added that was oxidized in five days was very much less than the percentage

TABLE VIII.

LAB. NO.	AMOUNT OF SULFUR ADDED	INCUBATION PERIOD	S AS SULFATE	S AS SULFATE (AVERAGE)	S AS SULFATE IN SOILS	S AS SULFATE OXIDIZED BY SOILS	SULFUR ADDED OXIDIZED
	gm.	*days*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
1	0.10	5	6.47				
2	"	5	6.75	6.61	3.93	2.68	2.68
3	"	7	13.35				
4	"	7	13.39	13.37	3.93	9.44	9.44
5	"	10	25.59				
6	"	10	25.19	25.39	3.93	21.46	21.46
7	"	14	33.75				
8	"	14	33.31	33.53	3.93	29.60	29.60
9	0.05	5	4.95				
10	"	5	5.19	5.07	3.93	1.14	2.28
11	"	7	8.00				
12	"	7	7.40	7.70	3.93	3.77	7.54
13	"	10	14.06				
14	"	10	13.95	14.00	3.93	10.07	20.15
15	"	14	17.44				
16	"	14	17.10	17.27	3.93	13.34	26.68
17	0.025	5	4.85				
18	"	5	4.67	4.76	3.93	0.83	3.32
19	"	7	6.44				
20	"	7	5.85	6.14	3.93	2.21	8.84
21	"	10	7.32				
22	"	10	7.03	7.17	3.93	3.24	12.96
23	"	14	10.30				
24	"	14	8.16	9.23	3.93	5.30	21.20

of the sulfur in the sodium sulfide oxidized in the same length of time. Evidently the free sulfur is oxidized much less rapidly than the sulfide.

Practically the same relations between the sulfofying powers of the various soils were found as when the sodium sulfide was used. It seems, therefore, that free sulfur would probably be a much better material to use than sodium sulfide.

Series VIII. Effect of time of incubation on extent of oxidation of free sulfur.

The experiments were performed like those of Series VII, except that the period of incubation was varied from five to fourteen days. The results in Table VIII show that with increasing periods of incubation there were increasing percentage oxidations in the case of all three amounts of sulfur, the gains being slightly larger in the case of the larger amount of sulfur. It is apparent from these results that the incubation period in sulfofication tests should be ten to fourteen days in duration where free sulfur is employed as a measure of the extent of the process, in order to permit accumulation of sufficient amounts of sulfates to bring out the maximum differences in sulfofying power among various soils.

Series IX. Effect of moisture.

Samples of air-dry soil were weighed out, sulfur was added as usual, 5 cc. of an infusion of fresh soil were introduced, and varying amounts of moisture applied. The samples were then incubated for ten days at room temperature. The results are given in Table IX. There was a gradual increase in sulfates produced with increasing amounts of water up to 25 per cent, and beyond that point a gradual decline, the amount of sulfates formed with 45 per cent water, approximately the saturation point, being just about the same as that formed with 5 per cent water.

This shows that when moisture conditions are at the optimum for the growth of crops sulfofication may occur to the optimum extent, other conditions being satisfactory. In other words, when the saturation point of a soil is 50 per cent, the optimum water content for the process of sulfofication of sulfur is 25 per cent. Furthermore it is evident that the saturation point of soils should be ascertained and the moisture content brought to the optimum for every soil tested in order to obtain an accurate determination of its sulfofying power. Two soils can not be compared as to sulfofying power without insuring the maintenance of optimum moisture conditions, and the actual percentages of water which this means may be quite widely separated.

TABLE IX.

LAB. NO.	WATER	S AS SULFATE	S AS SULFATE (AVERAGE)	S AS SULFATE IN SOILS	S AS SULFATE OXIDIZED BY SOILS	SULFUR ADDED OXIDIZED BY SOILS
	per cent	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
1	5.00	5.79				
2	"	5.59	5.69	4.58	1.11	1.11
3	10.00	6.89				
4	"	7.01	6.95	4.58	2.37	2.37
5	15.00	7.95				
6	"	7.63	7.79	4.58	3.21	3.21
7	25.00	12.54				
8	"	12.77	12.65	4.58	8.07	8.07
9	30.00	11.42				
10	"	11.08	11.25	4.58	6.67	6.67
11	35.00	10.80				
12	"	10.70	10.75	4.58	6.17	6.17
13	40.00	7.54				
14	"	9.40	8.47	4.58	3.89	3.89
15	45.00	6.12				
16	"	6.15	6.13	4.58	1.55	1.55

TABLE X.

LAB. NO.	SOIL USED	SAND USED	S AS SULFATE	S AS SULFATE (AVERAGE)	S AS SULFATE IN SOILS	S AS SULFATE OXIDIZED BY SOILS	SULFUR ADDED OXIDIZED BY SOILS
	gm.	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
1	100	—	19.06				
2	100	—	13.68	16.37	4.11	12.26	12.26
3	90	10	20.34				
4	90	10	21.32	20.83	3.70	17.13	17.13
5	80	20	23.80				
6	80	20	22.35	23.07	3.29	19.78	19.78
7	60	40	26.86				
8	60	40	26.36	26.61	2.47	24.14	24.14
9	50	50	28.66				
10	50	50	27.52	28.09	2.06	26.03	26.03
11	30	70	23.14				
12	30	70	22.13	22.63	1.23	21.40	21.40
13	20	80	19.10				
14	20	80	16.85	17.97	0.82	17.15	17.15
15	10	90	12.48				
16	10	90	Lost	12.48	0.41	12.07	12.07

Series X. Effect of aeration.

Air-dry soil and sand in varying proportions were weighed out in tumblers, sulfur was added to each, 10 cc. of an infusion of a fresh soil were introduced, and the moisture content was adjusted to the optimum, 25 per cent for the soil and 12 per cent for the sand. The samples were then incubated for ten days at room temperature, and the results secured upon their examination are recorded in Table X.

It is evident that the mixing of sand with the soil in gradually increasing amounts brings about a gradual increase in sulfate production up to a mixture of 50 per cent soil and 50 per cent sand, over twice as much sulfate being formed in the latter case. Increasing the amount of sand and consequently diminishing the soil, however, beyond this point brings about a gradual decline in sulfate production.

The effect of aeration in increasing sulfofication in soils is thus definitely shown. This increase was apparent up to the point where the humus content or the mineral matter content became a restricting factor of growth.

Series XI. Effect of carbohydrates.

As has been noted, Brioux and Guerbet studied the influence of carbohydrates on the oxidation of sulfur, and found that sugar and starch appreciably retarded the oxidation, while peptone and other nitrogenous substances favored it. In this work three carbohydrates were used: saccharose, which is readily soluble; starch, which is partially soluble; and filtered paper, ground fine, which is soluble only to a very slight extent. Samples of fresh soil were weighed off as usual, sulfur was added, and varying quantities of the various materials were added. The moisture content of the samples was adjusted to the optimum, using an additional amount where the larger quantities of the organic matter were added. The sulfur present as sulfate in the soil was determined, and the total sulfates produced at the end of ten days' incubation were ascertained. The plan of the tests and the results of the analyses are given in Table XI. It will be noted that no results are given where the 3 and 5 gram quan-

tities of saccharose were added. This is due to the fact that the sulfates produced stimulated the transformation of the sugar into organic acids. The extract secured upon shaking the soil with water for seven hours was consequently very dark in color, and when the barium chloride was added there was a large precipitation of barium salts of the organic acids. The 1 gram quantity of the sugar was not transformed sufficiently to inter-

TABLE XI.

SOIL NO.	ADDITION	S AS SULFATE	S AS SULFATE (AVERAGE)	S AS SULFATE IN SOIL	S AS SULFATE OXIDIZED BY SOILS	SULFUR ADDED OXIDIZED BY SOILS
		mg.	*mg.*	*mg.*	*mg.*	*per cent*
3	1 gm. saccharose	6.68				
4	" " "	7.70	7.19	4.42	2.77	2.77
5	3 gm. saccharose	—				
6	" " "	—	—	—	—	—
7	5 gm. saccharose	—				
8	" " "	—	—	—	—	—
9	1 gm. starch	11.12				
10	" " "	20.90	16.01	4.42	11.59	11.59
11	3 gm. starch	10.09				
12	" " "	17.39	13.74	4.42	9.32	9.32
13	5 gm. starch	9.88				
14	" " "	11.12	10.50	4.42	6.08	6.08
15	1 gm. filter paper	28.19				
16	" " "	26.78	27.48	4.42	23.06	23.06
17	3 gm. filter paper	15.44				
18	" " "	15.00	15.22	4.42	10.80	10.80
19	5 gm. filter paper	12.32				
20	" " "	12.40	12.36	4.42	7.94	7.94
21	Nothing	38.30				
22	"	38.30	38.30	4.42	33.88	33.88

fere with the precipitation, and the results obtained showed a depression in sulfofication. The addition of starch likewise depressed sulfofication; the larger the quantity added, the greater was the depression. Similarly with the additions of filter paper, the 1, 3, and 5 gram quantities all depressed the sulfofication, the largest amount giving the greatest depression in every case. It is interesting to note that the smallest amount of filter paper depressed the sulfofication only to a small extent, and that in nearly every case the largest amount depressed it less than the

smallest amount of saccharose or starch. In every case, too, the saccharose depressed the oxidation of the sulfur more than did the starch.

The results of these tests showed quite distinctly, therefore, that carbohydrates depressed the oxidation of sulfur, the larger the quantity the greater the depression. Furthermore, the soluble carbohydrates depressed the oxidation more than the insoluble. Thus, saccharose brought about the greatest retardation, the more insoluble starch caused a smaller retardation, and filter paper depressed to a still smaller degree.

CONCLUSIONS.

It is apparent from the results which have been discussed in the previous pages that the sulfofying power of soils may be determined in the laboratory. The method devised consists in adding a sulfide, preferably sodium sulfide, or free sulfur to fresh soil, adjusting the moisture content to the optimum, and incubating for five to ten days at room temperature. At the end of that time the sulfates are leached out by shaking with water for seven hours, precipitated with barium chloride, and determined by the use of the sulfur photometer.

The oxidation of sulfides and free sulfur in the soil was brought about mainly by bacterial agency. There was, however, a small chemical oxidation of the sulfides, Na_2S, K_2S, and CaS, in soil upon shaking for seven hours with water. The extent of this oxidation varied with different soils and sulfides, sodium sulfide showing the smallest change. There was practically no change in free sulfur upon shaking with water; hence this material is undoubtedly the best to use for sulfofication tests, although the incubation period must be continued for a longer time.

The slight chemical oxidation of sulfides upon shaking with water leads to the conclusion that there may be some chemical oxidation of sulfides in the soil. Thus, while the process of sulfofication is undoubtedly mainly bacterial, there may be some purely chemical action also.

The sulfofying power varied with the treatment previously undergone by the soils. The use of manure and of green manure

on soils increased their sulfofying power, and in general it appeared that soils poor in organic matter were low in sulfofication.

The water content of the soils also influenced the rate of oxidation of sulfur. Sulfofication was found to increase with increasing moisture until the optimum, or 50 per cent of the amount necessary for complete saturation, was reached. Beyond that point additions of water depressed sulfofication. This indicates that sulfofication may occur to the optimum extent where moisture conditions are at the optimum for plant growth.

Increasing the air content of the soil up to a certain point increased sulfofication; as mixing sand with soil up to 50 per cent of each increased sulfofication. Beyond that point a depression occurred, probably due to a lack of nutritive organic or mineral matter.

Additions of carbohydrates were found to depress sulfofication, the larger the amounts, the greater the depression; the depression varying also in the inverse ratio to the solubility of the carbohydrate material.

DOES BUTTER-FAT CONTAIN NITROGEN AND PHOSPHORUS?[1]

By THOMAS B. OSBORNE and ALFRED J. WAKEMAN.

(*From the Laboratory of the Connecticut Agricultural Experiment Station, New Haven.*)

(Received for publication, March 8, 1915.)

The fact that butter-fat promotes the normal growth of young rats, under dietary conditions in which lard and some other fats fail to do this, has raised the interesting question: To what constituent of the butter-fat is this due? Every fact, therefore, which will in any way aid in the difficult research to determine the nature of this substance is of much value. If, for example, it can be shown that butter-oil contains no nitrogen or phosphorus, or if it does contain minute, but distinctly smaller, quantities of these elements, than does the less potent butter-fat,[2] we can exclude from the list of possibilities all those substances which contain either nitrogen or phosphorus. Osborne and Mendel[2] have stated that they had been unable to find nitrogen or phosphorus in the butter-fat used in their feeding experiments, and concluded that the growth-promoting constituent did not contain these elements. McCollum and Davis[3] have questioned the absence of phosphorus and nitrogen from this fat, and say that McCollum[4] had been able nearly to free butter-fat from phosphorus only by washing with dilute hydrochloric acid.

After careful determinations made in this laboratory had failed to detect either nitrogen or phosphorus in our samples of butter-fat, we asked Dr. Otto Folin to check our negative results for nitrogen. This he kindly did and reported that: "I get a

[1] The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[2] T. B. Osborne and L. B. Mendel: this *Journal,* xx, p. 382, 1915.

[3] E. V. McCollum and M. Davis: *ibid.*, xix, p. 250, 1914.

[4] E. V. McCollum: *Am. Jour. Physiol.*, xxv, p. 120, 1909–10.

slight trace of nitrogen, 0.1 to 0.2 mg. from 10 grams of substance. This amount is so small as to be practically nil, especially as I am not prepared to say that a part of it did not get in with the reagents. I should not hesitate to call your purified butter nitrogen-free." Recently Funk and Macallum[5] have stated that they succeeded in obtaining nitrogen from centrifugated butter-fat. The amount found, if we understand them correctly, was equal to 31 mg. (16.8 + 14.0 mg.) from 12 kg. of butter, or 0.0003 per cent. Since this quantity is so small we consider that Funk and Macallum have demonstrated that butter-fat is practically free from nitrogen, and that definite conclusions cannot be drawn from the results of feeding experiments conducted under such conditions as are now possible, which are based on quantities of this order.

Dr. W. J. Gies, who has had a wide experience in determining phosphorus in organic substances, likewise offered to examine the butter-fat for traces of this element. He reported that: "Entirely negative results were obtained with quantities below 10 grams. Above that amount enough phosphorus was precipitated for gravimetric determination, although the yield of pyrophosphate was so small that the percentage figures show that only traces of phosphorus were present." From 21.3 grams of the butter-fat he obtained only 0.0038 gram of magnesium pyrophosphate, equal to 0.0049 per cent of phosphorus in the butter-fat.

It has been shown[6] that at least one-half of the butter-fat can be separated in the form of a solid fraction by crystallization from alcohol, and that this fraction entirely lacks the growth-promoting property, whereas the more soluble liquid fraction retains this property in a high degree. We accordingly have examined this potent liquid fraction, the "butter-oil," for phosphorus, with the idea that we should find more phosphorus in it than in the butter-fat, if the growth-promoting substance contains this element.

The butter-oil was prepared by saturating absolute alcohol at 40° with butter-fat which had previously been melted and centrifugated at high speed until perfectly clear. The solution was then cooled to −20° and filtered rapidly on a cold Buchner

[5] C. Funk and A. B. Macallum: *Ztschr. f. physiol. Chem.*, xcii, p. 13, 1914.

[6] Osborne and Mendel: *loc. cit.*

funnel and the filtrate concentrated *in vacuo* at 40° until the alcohol was entirely removed. Of this butter-oil 44.14 grams were divided among nine ordinary Kjeldahl nitrogen flasks and oxidized by boiling with sulphuric acid to which powdered ammonium nitrate was added from time to time. For this operation 440 cc. of sulphuric acid and 120 grams of ammonium nitrate were required. After the butter-oil was oxidized the solutions were brought together into one flask and concentrated to a volume of about 15 cc. On cooling, the residual solution solidified. The residue was dissolved in hot water, neutralized with ammonia, then acidified with nitric acid, 15 grams of ammonium nitrate were added, and 20 cc. of molybdic solution. After digesting for several hours at 65° the small precipitate of phosphomolybdate was filtered out, converted into ammonium magnesium phosphate and weighed as pyrophosphate, after standing forty hours. The amount was 12.5 mg., equal to 0.35 mg. of phosphorus.

Since a part of this phosphorus might have come from the reagents, a blank was run with the same quantities and under the same conditions. The magnesium pyrophosphate obtained was equal to 0.084 mg. of phosphorus. Deducting this we have 0.266 mg. of phosphorus from the 44.14 grams of butter-oil or 0.0006 per cent. Dr. Isidor Greenwald also kindly offered to determine phosphorus in this sample of butter-oil, using the method of Pouget and Chouchak.[7] He reported finding in duplicate determinations 0.000738 and 0.000726 per cent of phosphorus, results in close agreement with our own.

From these results we find that this sample of *butter-oil* contained at the most only one-sixth as much phosphorus as Dr. Gies found in the entire *butter-fat*, a quantity so small that it may have been due to an accidental contamination of the reagents. Since the growth-promoting substance is concentrated in the butter-oil we conclude from these experiments that if it does contain phosphorus or nitrogen, it is present in such minute amount as to make its isolation impossible. In view of the fact that butter contains about 15 per cent of butter-milk, which is rich in both phosphorus and nitrogen, it is far more

[7] I. Pouget and D. Chouchak: *Bull. soc. chim.*, series 4, v, p. 104, 1909; ix, p. 649, 1911.

probable that the traces of these elements which have been detected in the butter-fat are derived from that source. In fact it is surprising that so simple a process as centrifugation should remove the butter-milk so completely.

STUDIES ON THE PHYSIOLOGY OF REPRODUCTION IN THE DOMESTIC FOWL.

XIII. ON THE FAILURE OF EXTRACT OF PITUITARY BODY (ANTERIOR LOBE) TO ACTIVATE THE RESTING OVARY.

By RAYMOND PEARL and FRANK M. SURFACE.

(*From the Biological Laboratory of the Maine Agricultural Experiment Station, Orono. Paper No. 83.*)

(Received for publication, March 22, 1915.)

In an earlier paper we[1] have shown that the substance of the corpora lutea of the cow has the power to inhibit ovulation in an actively laying fowl. The next step in a biochemical analysis of the physiology of ovulation is clearly to determine, if possible, whether there is any chemical substance which will activate the resting ovary. If such a substance can be found we shall then have in hand the main features for the complete chemical control of the process of ovulation in the fowl. With such an activator it should then be possible to start and stop a hen's egg-laying activities at will.

It seemed to us that the most promising place to look, at the outstart, for a chemical activator of the resting ovary, would be in a secretion of some endocrinal gland. On the whole the most likely gland with which to begin the experiments seemed to be the pars anterior of the pituitary body. The connection of this gland with the genital organs has been well established.[2] The general effect of its secretion upon the gonads would appear from the work particularly of Aschner,[3] Goetsch and Cushing,[4]

[1] R. Pearl and F. M. Surface: this *Journal*, xix, pp. 263–278, 1914.

[2] S. Vincent: *Internal Secretion and the Ductless Glands*, London, 1912.

[3] B. Aschner: *Arch. f. d. ges. Physiol.*, cxlvi, pp. 1–146, 1912.

[4] E. Goetsch and H. Cushing: *Proc. Soc. Exper. Biol. and Med.*, xi, pp. 26–27, 1913.

and Adler[5] clearly to be in the direction of activation or stimulation. Accordingly the substance of this gland was chosen as the point of departure in the present work.

The only long continued and certain period of rest of the ovary in the domestic fowl is that associated with the autumn moult of adult birds. The periods of rest intervening between laying cycles of shorter or longer duration are of too uncertain length to be of any critical use in studies like the present one. In many cases during these minor cyclical periods of rest, as indicated by absence of egg production, the ovary does not actually come into anything like a condition of physiological rest. It is only producing yolk at a very much reduced rate. This is very clearly shown in curves made by a new method[6] of measuring rate of fecundity in the individual, and has been discussed in earlier papers from this laboratory by the present writers[7] and Curtis.[8]

On the other hand Gerhartz[9] has shown by direct metabolism measurements that the period of moult is a period of very profound physiological depression of the whole organism. Here we are dealing with a condition of true physiological rest. Accordingly this is the period which has been chosen for the present study. The birds used were well along in moult; that is, the shedding of the old and the growth of the new feathers were well started. But, in the ordinary course of events, these birds would not, in most instances, have laid any eggs until at least one to two months later. If then laying should promptly follow the injection of pituitary substance in such cases it could be safely concluded that it was because of the injection. No really critical results could be obtained with hens at other times in the year, since if a non-laying hen, in a pseudo-rest period between cycles, began to lay immediately after injection, one could not assert that she would not have done the same in the absence of any injection whatever.

[5] L. Adler: *Arch. f. Entwcklngsmechn. d. Organ.*, xxxix, pp. 21–45, 1914.

[6] R. Pearl: *Science*, xl, pp. 383–384, 1914.

[7] Pearl and Surface: *U. S. Department of Agriculture, Bureau of Animal Industry, Bull. 110*, pt. ii, 1911.

[8] M. R. Curtis: *Arch. f. Entwcklngsmechn. d. Organ.*, xxxix, pp. 217–327, 1914.

[9] H. Gerhartz: *Arch. f. d. ges. Physiol.*, clvi, pp. 1–224, 1914.

EXPERIMENTAL.

The pituitary substance used in these experiments was obtained from the Organotherapeutic Laboratory of Armour and Company, in Chicago. Two different lots of the substance were used. One, here called A, was obtained in November 1913, and was approximately a year old when used. The other, here called B, was obtained early in November 1914, through the kindness of Dr. Frederic Fenger, to whom we are greatly indebted for the painstaking care used in the preparation of this and other organ substances for use in our experiments, and was used when less than one month old, counting from the living animal.

The method of collecting and preparing the glands was as follows:[10]

"The pituitary bodies were removed from the animals immediately after slaughtering and chilled. The glands were carefully freed from all adhering connective tissue and the posterior lobes with the infundibular stalk dissected out. The anterior lobes were then rinsed with water to remove blood and possible traces of posterior secretion, finely minced and dried. The desiccation was carried on at about 35°C., until approximately 80 per cent of the moisture had been driven off. The temperature was then gradually raised to about 50° C., which is well below the coagulating temperature of the proteids.

"The desiccated material was coarsely ground and extracted with petroleum ether in a Soxhlet Extractor. The desiccated fat-free material was then ground in a steel tube mill to pass an 80 mesh sieve."

The birds used were all normal, healthy, yearling fowls, in the process of moulting at the end of their first or pullet laying year.

The experimental results are shown in the following tables.

But little comment on these tables is necessary so far as concerns the main point here under consideration. It is clear that the *pituitary body substance induced no activation of the completely resting ovary.* On the contrary, what influence it had on the ovary was *apparently* in the direction of lengthening somewhat the rest period, but with so few birds a definite conclusion on this point is impossible. That a positive activating effect did *not*

[10] F. Fenger: Personal communication, Nov. 11, 1914.

TABLE 1.

Series I. Single intra-abdominal injection. Substance A.

BIRD NO.	BODY WEIGHT IN GM.	DATE OF INJECTION	DOSE* OF DRY SUBSTANCE IN GM.	CONDITION AS TO MOULT AT TIME OF INJECTION	DATE OF LAST EGG BEFORE INJECTION	DATE OF FIRST EGG AFTER INJECTION	DURATION OF REST PERIOD IN DAYS	DAYS FROM INJECTION TO FIRST EGG	REMARKS
151	2170	Oct. 15, 1914	0	Late stage	Sept. 4	Jan. 12	130	89	Control. Got 15 cc. salt sol. Operated Nov. 16. See text.
142	1780	" " "	0.5	Middle "	Aug. 24	Feb. 8	168	116	Operated Nov. 16. See text.
133	2260	" " "	1.0	" "	Aug. 27	Dec. 13	108	59	Autopsied Dec. 15. " "
181	2030	" " "	1.5	Early "	Sept. 25	Dec. 18	84	64	
165	2380	" " "	2.0	" "	Sept. 29	Feb. 11	135	119	Operated Nov. 24.

* In this and all of the other experiments the amount of dry substance indicated in the tables was mixed, in every case, with 15 cc. sterile 0.9 per cent NaCl solution, warmed to body temperature, at the time of injection.

TABLE 2.

Series II. Single intra-abdominal injection. Substance B.

BIRD NO.	BODY WEIGHT IN GM.	DATE OF INJECTION	DOSE OF DRY SUBSTANCE IN GM.	CONDITION AS TO MOULT AT TIME OF INJECTION	DATE OF LAST EGG BEFORE INJECTION	DATE OF FIRST EGG AFTER INJECTION	DURATION OF TOTAL REST PERIOD IN DAYS	DAYS FROM LAST INJECTION TO FIRST EGG	REMARKS
110	2620	Nov. 25, 1914	0	Late stage	Sept. 28	Nov. 28	61	3	Control. NaCl only.
8065	2730	" " "	0.5	" "	Oct. 13	Dec. 16	64	21	
112	3120	" " "	1.0	Middle "	Sept. 28	Later than Mar. 14	Over 167	Over 109	
124	1980	" " "	1.5	" "	Sept. 29	" " Feb. 10	" 134	" 77	Disposed of on date mentioned, because of lack of space.
8076	2890	" " "	2.0	" "	Aug. 14	——	——	——	Died Nov. 26. See text.

TABLE 3.

Series III. Repeated intra-abdominal injections. Substance A.

Bird No.	First injection Oct. 24, 1914		Second injection Oct. 30, 1914		Third (or second) injection Nov. 5, 1914		Condition as to moult at time of first injection	Date of last egg before injection	Date of first egg before injection	Duration of total rest period in days	Days from last injection to first egg	Remarks
	Body weight in gm.	Dose of dry substance in gm.	Body weight in gm.	Dose of dry substance in gm.	Body weight in gm.	Dose of dry substance in gm.						
131	2540	0.5	2410	—*	2550	0.5	Late stage	July 22	Later than Mar. 14	Over 235	Over 129	
153	2100	1.0	1950	1.0	1950	1.0	Middle "	Oct. 5	Dec. 29	85	54	Operated Nov. 16.
1424	1500	1.5	1590	—*	1740	1.5	" "	Aug. 22	——	—	—	Autopsied Nov. 12.
137	2300	2.0	2140	—*	2040	2.0	" "	Aug. 22	Feb. 11	173	98	

* The only available syringe broke after the injection of No. 153 on this day so that these birds got none.

follow the injection may however be concluded with entire certainty.

The immediate effect of the pituitary substance was to induce rapid and difficult breathing during the first twenty-four hours after the injection. This effect was most marked in Series II where the fresh substance was used. All of the birds in this series were plainly ill on the day following the injection, and one (8076) died. The immediate effects were in proportion to the size of the dose. Undoubtedly there were associated blood pressure effects but no attempt was made to measure these.

Bird No. 1424 (Series III) was killed and autopsied a week after the last injection to determine, by direct examination of the ovary, whether anything like a process of activation was going on.

Autopsy Record: Bird No. 1424. Killed by bleeding, 3 p.m., Nov. 12, 1914.

Bird entirely normal. No trace of peritonitis. The injected pituitary substance was found in small lumps scattered about among the viscera, walled off by peritoneum, and undergoing absorption. Ovary and oviduct entirely *normal* but completely in dormant or *inactive* condition. Ovary had a few old absorbing yolks, of small size (smaller than a pea). These probably date back to the cessation of laying. Oviduct inactive. No trace of peritonitis. Heart, spleen, kidneys *normal.*

Bird No. 133 was killed and autopsied Dec. 15, 1914, after she had begun to lay. Small masses of hard material were found scattered about the peritoneum, and undoubtedly represented the final remains of the pituitary substance in process of absorption.

In the case of the birds marked "Operated" in the tables, the abdominal cavity was opened under ether anesthesia on the date mentioned, the ovary examined, and a portion of it removed. In every case except one the ovary was in complete resting condition at the date of operation. In the exceptional case (bird No. 165) the ovary was in laying condition and the bird would have produced an egg within a few days if no operative disturbance had intervened. The time in the case had, however, been too long to think of activation having resulted from the injection. The extensive studies on the function of egg produc-

tion in poultry which have been carried out in this laboratory would indicate that if one is to conclude, with any critical safety, that activation of the ovary has resulted from the administration of any particular chemical substance, this activation must show clear evidence of itself within a period, at the outside, of fourteen or fifteen days after its administration.

CONCLUSION.

From the evidence presented in this paper it appears to be clearly established that the substance of the anterior lobe of the pituitary body of the cow, when injected into the abdominal cavity of hens in which the ovary is in a completely resting condition, does not cause an activation of the ovary, in the sense of inducing ovulation at an earlier date than that at which it would normally occur.

THE QUATERNARY SALTS OF HEXAMETHYLENE-TETRAMINE.

III. MONOHALOGENACYLATED AROMATIC AMINES AND THEIR HEXAMETHYLENETETRAMINIUM SALTS.[1]

By WALTER A. JACOBS and MICHAEL HEIDELBERGER.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

(Received for publication, March 29, 1915.)

In the second of this series of papers we have described a number of compounds obtained from the halogenacetyl derivatives of various benzylamines. The present paper will deal with those obtained from the halogenacetyl derivatives of aromatic amines in which the amino group is situated directly on the nucleus. The scope of the work and the great variety of amino compounds employed demonstrate how practically limitless in this direction is the development of hexamethylenetetraminium salts.

As described in greater detail in the first paper, the addition of the halogen compound to hexamethylenetetramine was accomplished by the use of molecular equivalents of the constituents in a small volume of dry, boiling chloroform. Unless otherwise stated the products separated during the reaction and were filtered off and washed well with dry chloroform and dry acetone. Purity of the final product is assured by the use of pure materials. Otherwise it is, as a rule, difficult or impossible to recrystallize the salts without danger of decomposition. These substances are usually readily soluble in water, in which, particularly on boiling, they decompose with separation of precipitates. These, on purification, proved to be the methyleneglycyl derivatives of the amines employed. A description of these substances and a discussion of their significance is reserved for a later communication.

[1] For parts I and II of this series see this *Journal*, xx, pp. 659 and 685, 1915.

In the course of the work occasion arose for the preparation of new intermediate products which will be described at length in the experimental part.

EXPERIMENTAL.

The method most frequently used for the chloroacetylation was as follows: One molecular equivalent of the base was dissolved in toluene or benzene. To the solution was added an excess of two-normal sodium hydroxide solution, and then, drop by drop, with cooling and vigorous shaking, a solution of one and one-quarter molecules of chloroacetyl chloride in about two volumes of dry benzene. On short standing, the chloroacetyl derivative usually crystallized out, the precipitation being facilitated in a number of cases by the addition of ligroin. In dealing with the more soluble and more easily accessible amines, two molecules of base to one of chloride were used, thus dispensing with the need of the aqueous alkali solution. Important deviations from the above method are indicated as they occur.

Chloroacetylaniline and hexamethylenetetramine. Equimolecular quantities of the components were boiled in dry chloroform solution for one-half hour. The salt separated on chilling and scratching, and was obtained in practically quantitative yield on completing the precipitation with an equal volume of dry acetone. It forms glistening micro-crystals, readily soluble in the cold in water and absolute alcohol. It darkens at 155° and melts with decomposition at 158–9°.

0.2987 gm. of substance required 9.6 cc. $AgNO_3$ Solution I.[2]
Calculated for $C_{14}H_{20}ON_5Cl$: Cl = 11.45 per cent.
Found: Cl = 11.31 per cent.

Bromoacetylaniline and hexamethylenetetramine. The solution of the components in boiling chloroform soon set to a solid cake. Heated in a capillary tube, the salt first darkens, then melts at 163° with decomposition. It is less soluble than the chloride.

0.2043 gm. of substance required 5.8 cc. $AgNO_3$ Solution I.
Calculated for $C_{14}H_{20}ON_5Br$: Br = 22.55 per cent.
Found: Br = 22.52 per cent.

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[2] 1 cc. = 0.00352 gm. Cl; 0.00793 gm. Br; 0.01259 gm. I.

Chloroacetylmethylaniline was found to have the melting point given by Grothe[3] (70°), and not that recorded by Kuhara and Chikashigé[4] (61°) or Bischoff[5] (48°).

Chloroacetylmethylaniline and hexamethylenetetramine. The chloroform solution was evaporated to small bulk after two hours' boiling, and the salt precipitated by adding dry acetone. Three extractions of one hour each with boiling, dry acetone were found necessary to remove the chloroform of crystallization from the product. It melts at 168–73° with gas evolution, and is easily soluble in chloroform and alcohol. It is extremely soluble in water.

0.1984 gm. of substance required 6.3 cc. $AgNO_3$ Solution I.
Calculated for $C_{15}H_{22}ON_5Cl$: Cl = 10.96 per cent.
Found: Cl = 11.18 per cent.

Chloroacetyldiphenylamine[6] *and hexamethylenetetramine.* The salt, as prepared in chloroform solution, forms hair-like needles, decomposing at 158–61°, and retaining a molecule of chloroform of crystallization even at 100° *in vacuo.*

0.2037 gm. of substance required 4.01 cc. $AgNO_3$ Solution I.
Calculated for $C_{20}H_{24}ON_5Cl \cdot CHCl_3$: Cl = 7.02 per cent.
Found: Cl = 6.93 per cent.

An attempt to remove the chloroform by boiling the salt with dry acetone led to the substitution of two molecules of acetone for the molecule of chloroform, the resulting product decomposing at 151–62°.

0.2075 gm. of substance required 4.12 cc. $AgNO_3$ Solution I.
Calculated for $C_{20}H_{24}ON_5Cl \cdot 2CH_3COCH_3$: Cl = 7.07 per cent.
Found: Cl = 6.99 per cent.

This conclusion was confirmed by the absence of the odor of chloroform on dissolving the substance in water, and by the fact that the distillate from the aqueous solution yielded a copious precipitate of iodoform, negative results being obtained on con-

[3] W. Grothe: *Chem. Zentralbl.*, lxxi, pt. ii, p. 1268, 1900.
[4] M. Kuhara and M. Chikashigé: *Am. Chem. Jour.*, xxvii, p. 1, 1902.
[5] C. A. Bischoff: *Ber. d. deutsch. chem. Gesellsch.*, xxxiv, p. 2125, 1901.
[6] H. Frerichs: *Chem. Zentralbl.*, lxxiv, pt. ii, pp. 103–4, 1903.

trols run with chloroform and the hexamethylenetetraminium salt of chloroacetylaniline.

Chloroacetylphenylglycinanilide. The benzene layer from the chloroacetylation of the base was evaporated to a syrup *in vacuo*, water added, and the remaining benzene removed by further distillation. The water was poured off from the tarry residue, which was taken up in alcohol and made to crystallize by the cautious addition of water. Yield: poor. The substance was recrystallized twice from 95 per cent alcohol, then twice from benzene, from which it separates with benzene of crystallization. This is given off very slowly *in vacuo* at room temperature. After finally heating at 100°, the compound melts at 118–20° (corrected) with preliminary softening. It is readily soluble in chloroform, difficultly in ether.

0.1461 gm. of substance (Kjeldahl) required 9.45 cc. $\frac{N}{10}$ HCl.
0.2010 gm. of substance[7] required 12.50 cc. $AgNO_3$ Solution II.[8]
Calculated for $C_{16}H_{15}O_2N_2Cl$: N = 9.26 per cent; Cl = 11.72 per cent.
Found: N = 9.06 per cent; Cl = 11.57 per cent.

Chloroacetylphenylglycinanilide and hexamethylenetetramine. The presence of a little chloroform in the salt, even after drying to constant weight *in vacuo* at the temperature of boiling alcohol over sulphuric acid, was recognized by its swimming about on solution in water, and by the odor evolved in so doing. The salt melts at 157–8°.

0.1939 gm. of substance required 7.83 cc. $AgNO_3$ Solution II.
Calculated for $C_{22}H_{27}O_2N_6Cl$: Cl = 8.10 per cent.
Found: Cl = 7.51 per cent.

Chloroacetyl-ω-anilinoacetophenone. 6.8 grams of anilinoacetophenone (phenacylaniline) were suspended in about 25 cc. of benzene and 3.8 grams of chloroacetyl chloride added. After the first evolution of heat, the mixture was warmed on the water bath for two hours, hydrochloric acid being evolved. On cooling, a small residue was filtered off, and the filtrate treated with ligroin, precipitating an oil which rapidly solidified. This was recrystallized from dilute alcohol, forming prisms with a slight

[7] Hydrolysis with alcoholic sodium hydroxide.
[8] 1 cc. = 0.00186 gm. Cl; 0.004192 gm. Br.

greenish yellow tinge and melting at 116–7.5°. Yield: 7.3 grams. Recrystallized again from methyl alcohol, the substance forms practically colorless, hexagonal plates, melting at 117–8° (corrected) with slight softening at 116.5°. It is difficultly soluble in cold methyl alcohol and dry ether.

0.3579 gm. of substance (Kjeldahl) required 12.45 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{16}H_{14}O_2NCl$: N = 4.87 per cent.
Found: N = 4.87 per cent.

Chloroacetyl-ω-anilinoacetophenone and hexamethylenetetramine. The salt is somewhat soluble in chloroform, easily in water and absolute alcohol, and melts with decomposition at 169–9.5°.

0.2246 gm. of substance required 5.41 cc. $AgNO_3$ Solution I.
Calculated for $C_{22}H_{26}O_2N_5Cl$: Cl = 8.29 per cent.
Found: Cl = 8.48 per cent.

Chloroacetyl-o-toluidine and hexamethylenetetramine. The salt melts with decomposition at 148–9° and is easily soluble in water.

0.3189 gm. of substance required 9.85 cc. $AgNO_3$ Solution I.
Calculated for $C_{15}H_{22}ON_5Cl$: Cl = 10.96 per cent.
Found: Cl = 10.87 per cent.

Bromoacetyl-ω-o-toluidinoacetophenone. 14.6 grams of phenacyl-*o*-toluidine were dissolved in about 100 cc. of benzene and 15 grams of bromoacetyl bromide added. The reaction occurred at once with separation of the hydrobromide of the base. On boiling for three-quarters of an hour, hydrobromic acid was evolved and a clear solution obtained. About two-thirds of the benzene were then removed and the bromoacetyl derivative was precipitated by the addition of ligroin to the warm solution. After recrystallization from 85 per cent alcohol it formed thick, diamond-shaped plates melting at 107–7.5°. Yield: 17.3 grams. The compound is extremely soluble in chloroform, less so in benzene, and difficultly in ether.

0.3361 gm. of substance (Kjeldahl) required 9.9 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{17}H_{16}O_2NBr$: N = 4.05 per cent.
Found: N = 4.13 per cent.

Bromoacetyl-ω-o-toluidinoacetophenone and hexamethylenetetramine. On heating a chloroform solution of the components to

boiling, the reaction proceeded spontaneously with formation of a clear solution. After one-half hour's heating, a small amount of what was probably hexamethylenetetraminium bromide was filtered off and an equal volume of dry acetone added. The salt was then precipitated as a caseous mass by means of dry ether, becoming crystalline on rubbing. It was purified by warming with dry acetone, in which it proved to be insoluble when once crystalline. The salt melts with decomposition at 175–6° and is difficultly soluble in water and chloroform.

0.2017 gm. of substance required 4.24 cc. $AgNO_3$ Solution I.
Calculated for $C_{23}H_{28}O_2N_5Br$: Br = 16.43 per cent.
Found: Br = 16.68 per cent.

Chloroacetyl-*m*-toluidine was prepared from *m*-toluidine in toluene, normal potassium hydroxide solution, and chloroacetyl chloride. The compound separated in good yield on adding ligroin and cooling. Whereas Kuhara and Chikashigé[4] give 141° as the melting point, our preparation, after recrystallization first from dilute alcohol then toluene, melted at 90–1.5° (corrected).

0.2550 gm. of substance (Kjeldahl) required 13.80 cc. $\frac{N}{10}$ HCl.
Calculated for $C_9H_{10}ONCl$: N = 7.63 per cent.
Found: N = 7.58 per cent.

Chloroacetyl-m-toluidine and hexamethylenetetramine. After short boiling, the chloroform solution of the components suddenly set to a solid cake. This was disintegrated and boiled for ten minutes longer with a little more dry chloroform. The salt darkens above 170° and melts at 173–4°. It is very soluble in water.

0.1622 gm. of substance required 9.10 cc. $AgNO_3$ Solution II.
Calculated for $C_{15}H_{22}ON_5Cl$: Cl = 10.96 per cent.
Found: Cl = 10.43 per cent.

Chloroacetyl-p-toluidine and hexamethylenetetramine. As obtained from chloroform solution, the salt was not entirely soluble in water. It was purified by boiling with dry acetone, whereupon it dissolved completely, although rather difficultly, in water. It melts at 176–8° with decomposition.

0.2744 gm. of substance required 8.36 cc. $AgNO_3$ Solution I.
Calculated for $C_{15}H_{22}ON_5Cl$: Cl = 10.96 per cent.
Found: Cl = 10.72 per cent.

Chloroacetyl-m-4-xylidine[4] *and hexamethylenetetramine.* The salt forms silky needles, melting at 171–2°.

0.2170 gm. of substance required 6.3 cc. $AgNO_3$ Solution I.
Calculated for $C_{16}H_{24}ON_5Cl$: Cl = 10.52 per cent.
Found: Cl = 10.22 per cent.

Chloroacetyl-ψ-cumidine[4] *and hexamethylenetetramine.* The salt separated from chloroform in fine needles, and was purified by boiling with hot ethyl acetate. It melts with decomposition at 170–1° and is easily soluble in water and alcohol.

0.2183 gm. of substance required 6.14 cc. $AgNO_3$ Solution I.
Calculated for $C_{17}H_{26}ON_5Cl$: Cl = 10.08 per cent.
Found: Cl = 9.90 per cent.

Chloroacetyl-α-naphthylamine and hexamethylenetetramine. On adding several volumes of dry acetone to the chloroform reaction mixture, the salt separated after a few moments in rosettes of needles, melting at 164–6° with decomposition and difficultly soluble in water.

0.2849 gm. of substance required 7.97 cc. $AgNO_3$ Solution I.
Calculated for $C_{18}H_{22}ON_5Cl$: Cl = 9.86 per cent.
Found: Cl = 9.85 per cent.

Chloroacetyl-β-naphthylamine[9] *and hexamethylenetetramine.* The salt melts at 177–8° with decomposition and is difficultly soluble in water.

0.2612 gm. of substance required 7.15 cc. $AgNO_3$ Solution I.
Calculated for $C_{18}H_{22}ON_5Cl$: Cl = 9.86 per cent.
Found: Cl = 9.64 per cent.

Chloroacetyl-*o*-chloroaniline was prepared by Schwalbe, Schulz, and Jochheim[10] by two methods differing from the one outlined

[9] T. B. Johnson and W. K. Walbridge: *Jour. Am. Chem. Soc.*, xxv, p. 483, 1903.
[10] C. G. Schwalbe, W. Schulz, and H. Jochheim: *Ber. d. deutsch. chem. Gesellsch.*, xli, p. 3791, 1908.

above. After chloroacetylation, the toluene layer was washed successively with dilute hydrochloric acid, water, dilute sodium hydroxide, and water, and evaporated to small bulk. The substance separated as glistening needles after the addition of ligroin. Recrystallized from benzene, it softens at 71.5° and melts at 73.5–4.° (corrected). The above mentioned authors give 67° as the melting point.

Chloroacetyl-o-chloroaniline and hexamethylenetetramine. The salt separates from the boiling chloroform solution in the form of glistening hairs. It melts with decomposition at 158–60° and is somewhat difficultly soluble in water.

0.1722 gm. of substance required 9.37 cc. $AgNO_3$ Solution II.
Calculated for $C_{14}H_{19}ON_5Cl_2$: $Cl^- = 10.30$ per cent.
Found: $Cl^- = 10.12$ per cent.

p-Bromochloroacetylaniline. This was prepared by Frerichs[6] from *p*-bromoaniline. We have found that the substance is easily obtained by treating a solution of chloroacetylaniline in acetic acid drop by drop with one molecular equivalent of bromine dissolved in acetic acid. The bromo derivative separates out in part on standing, and is more completely precipitated on dilution of the solvent with water. After recrystallization from acetic acid or dilute alcohol, it conforms to the description given by Frerichs.

p-Bromochloroacetylaniline and hexamethylenetetramine. In order to obtain a product entirely soluble in water, it was necessary to boil out the crude salt with a fresh portion of chloroform. It forms prismatic needles containing chloroform of crystallization, a part of which is retained even on drying to constant weight *in vacuo* at the temperature of boiling alcohol over sulphuric acid, or on boiling out with dry acetone.

0.1783 gm. of substance (Kjeldahl) required 20.54 cc. $\frac{N}{10}$ HCl.
0.2476 gm. of substance required 5.59 cc. $AgNO_3$ Solution I.
Calculated for $C_{14}H_{19}ON_5ClBr \cdot \frac{2}{5}CHCl_3$: N = 16.05 per cent; Cl = 8.13%.
Found: N = 16.13 per cent; Cl = 7.95%.

The original crystals apparently contained $\frac{4}{5}$ $CHCl_3$, as a portion, dried between filter papers, lost 9.47 per cent on drying to constant weight as above. Calculated for $\frac{2}{5}CHCl_3$: Loss = 9.87 per cent.

2, 4, 6-Tribromochloroacetylaniline. 9 grams of tribromoaniline and 2.7 cc. of chloroacetyl chloride were heated in about 100 cc. of toluene in an oil bath at 110–20° for about four hours. On cooling, the deposit of needles was filtered off and recrystallized from xylene. Yield: 8.5 grams. The substance melts at 221–2° (corrected) with partial decomposition, and is difficultly soluble, in the cold, in the usual solvents.

0.3350 gm. of substance (Kjeldahl) required 8.1 cc. $\frac{N}{10}$ HCl.
Calculated for $C_8H_5ONClBr_3$: N = 3.45 per cent.
Found: N = 3.39 per cent.

An attempt to prepare the hexamethylenetetraminium salt resulted only in the isolation of a product consisting largely of hexamethylenetetraminium chloride.

m-Iodochloroacetylaniline. The substance separated from the toluene solution when prepared as described in the introduction. It forms slender, felted needles, which, when recrystallized first from dilute alcohol, then toluene, melt at 121.5–2.5° (corrected). It is fairly readily soluble in cold absolute alcohol, benzene, and chloroform.

0.3534 gm. of substance (Kjeldahl) required 12.05 cc. $\frac{N}{10}$ HCl.
Calculated for C_8H_7ONClI: N = 4.74 per cent.
Found: N = 4.77 per cent.

m-Iodochloroacetylaniline and hexamethylenetetramine. The salt turns greenish above 170° and melts with decomposition at 175°. It is difficultly soluble in water.

0.1922 gm. of substance required 8.08 cc. $AgNO_3$ Solution II.
Calculated for $C_{14}H_{19}ON_5ClI$: Cl = 8.14 per cent.
Found: Cl = 7.82 per cent.

5-Iodochloroacetyl-o-toluidine. This was prepared from 5-iodo-2-toluidine[11] similarly to the above aniline derivative. Recrystallized first from dilute alcohol, then from chloroform, it forms glistening needles melting at 161.5–2.5° (corrected) with slight preliminary softening. It is more soluble in the cold in ethyl acetate, absolute alcohol, and acetone than in the other usual organic solvents.

[11] H. L. Wheeler and L. M. Liddle: *Am. Chem. Jour.*, xlii, p. 501, 1909.

0.3185 gm. of substance (Kjeldahl) required 10.20 cc. $\frac{N}{10}$ HCl.
Calculated for C_9H_9ONClI: N = 4.53 per cent.
Found: N = 4.48 per cent.

5-Iodochloroacetyl-o-toluidine and hexamethylenetetramine. Combination of the components in boiling chloroform proceeded slowly. The salt forms minute, flat prisms which melt with decomposition at 190–2° and are rather difficultly soluble in water.

0.2045 gm. of substance required 8.37 cc. $AgNO_3$ Solution II.
Calculated for $C_{15}H_{21}ON_5ClI$: Cl = 7.89 per cent.
Found: Cl = 7.61 per cent.

m-Nitrochloroacetylaniline[12] *and hexamethylenetetramine.* The pale yellow salt melts at 162–3°.

0.2264 gm. of substance required 6.3 cc. $AgNO_3$ Solution I.
Calculated for $C_{14}H_{19}O_3N_6Cl$: Cl = 10.00 per cent.
Found: Cl = 9.8 per cent.

p-Nitrochloroacetylaniline. 24 grams of commercial *p*-nitraniline were dissolved in commercial acetone and filtered from impurities. 7.4 cc. of chloroacetyl chloride were then slowly added, with stirring and cooling. Without removing the precipitated hydrochloride, dilute hydrochloric acid was added in excess, and then, to the resulting clear solution, hot water until crystallization started. On cooling rapidly, the chloroacetyl derivative separated in glistening crystals. Yield: about 90 per cent of the theory. Recrystallized from acetic acid, it forms pale brownish yellow prisms which melt at 182–4° with preliminary softening. Deutsch[13] describes the substance as sintering above 124° and melting at 152°.

0.4770 gm. of substance (Kjeldahl) required 43.95 cc. $\frac{N}{10}$ HCl.
Calculated for $C_8H_7O_3N_2Cl$: N = 13.06 per cent.
Found: N = 12.90 per cent.

m-Nitrochloroacetyl-p-toluidine and hexamethylenetetramine. The pale yellow salt melts with decomposition at 163–4° and turns bright yellow when added to water, dissolving slowly and giving a yellow solution. The salt is also somewhat soluble in alcohol.

[12] T. B. Johnson and W. B. Cramer: *Jour. Am. Chem. Soc.*, xxv, p. 490, 1903.

[13] E. Deutsch: *Jour. f. prakt. Chem.*, lxxvi, p. 360, 1907.

0.2368 gm. of substance required 6.49 cc. $AgNO_3$ Solution I.
Calculated for $C_{15}H_{21}O_3N_6Cl$: Cl = 9.59 per cent.
Found: Cl = 9.65 per cent.

m-Chloroacetylaminodimethylaniline. Groll's[14] method for the preparation of *m*-nitrodimethylaniline proved to be very unsatisfactory, the *p*-isomer being obtained as the chief product.

m-Aminodimethylaniline (Schuchardt) was chloroacetylated in benzene solution. In order to complete the separation of the chloroacetyl derivative, about one-half volume of ligroin was added to the benzene layer and the mixture allowed to stand over night in the ice box. Yield: 50–80 per cent of the theory. After two recrystallizations from 95 per cent alcohol, the compound forms grayish rhombs, softening at 101° and melting at 101.5–2.5° (corrected). It couples readily with diazo compounds, and is very soluble in the cold in acetone, less so in acetic acid, and difficultly in alcohol, benzene, and ether.

0.1614 gm. of substance (Kjeldahl) required 15.2 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{10}H_{13}ON_2Cl$: N = 13.18 per cent.
Found: N = 13.19 per cent.

m-Chloroacetylaminodimethylaniline and hexamethylenetetramine. The green, viscous solution obtained by boiling the components in dry chloroform was diluted with several volumes of dry acetone, precipitating the salt as a pale bluish, microcrystalline powder. Boiling for one hour in dry acetone removed most of the color. The purified salt melts with decomposition at 151–2° and is readily soluble in water, but difficultly in chloroform.

0.2220 gm. of substance required 6.59 cc. $AgNO_3$ Solution I.
Calculated for $C_{16}H_{25}ON_6Cl$: Cl = 10.05 per cent.
Found: Cl = 10.45 per cent.

p-Aminodimethylaniline. A portion of the *p*-aminodimethylaniline used in this and other experiments was furnished us through the courtesy of the Farbwerke Höchst. We desire to take this opportunity also to thank this firm for their kindness in supplying us with this and other intermediate products of the dyestuff industry. In preparing additional quantities of *p*-aminodi-

[14] A. Groll: *Ber. d. deutsch. chem. Gesellsch.*, xix, p. 198, 1886.

methylaniline, the following method was found to be very rapid and serviceable: 100 grams of *p*-nitrosodimethylaniline were added in small amounts to a warm solution of 450 grams of stannous chloride in 900 cc. of concentrated hydrochloric acid, occasional cooling being necessary. The mixture was finally warmed for one-half hour on the water bath, cooled, and saturated with hydrochloric acid gas at 0° in order to complete the precipitation of the double tin salt. This was filtered off, dissolved in water, the solution covered with a layer of ether, and, with cooling, made very strongly alkaline with 50 per cent sodium hydroxide solution. The upper layer was removed and the alkaline solution shaken out several times with ether. After removal of the ether, the residue was fractionated *in vacuo*. Yield: 72 grams, boiling at 146–8° at 24 mm., and melting at 38–41°.

p-Chloroacetylaminodimethylaniline. After chloroacetylation of the base in benzene solution, using chloroacetyl chloride and aqueous sodium hydroxide, the separation of the chloroacetyl derivative was completed by the addition of ligroin. Recrystallized from benzene, with bone-blacking, the compound answered the description given by Rupe and Vśetečka.[15] Yield: 74 per cent of the theory.

p-Chloroacetylaminodimethylaniline and hexamethylenetetramine. The salt forms glistening plates containing chloroform of crystallization and melting at 145–8°. After drying to constant weight *in vacuo* at the temperature of boiling alcohol, the substance melts at 171–2° with decomposition and preliminary darkening, and still retains chloroform. It is easily soluble in water, the solution smelling of chloroform and giving an orange-red color with ferric chloride. During the analysis by the Volhard method, a deep pink color developed, but it was found possible to destroy this by adding a large excess of concentrated nitric acid, thus permitting the determination of the end-point.

0.2100 gm. of substance required 5.23 cc. $AgNO_3$ Solution I.
Calculated for $C_{16}H_{25}ON_6Cl \cdot \frac{1}{2}CHCl_3$: $Cl^- = 8.60$ per cent.
Found: $Cl^- = 8.77$ per cent.

p-Aminodiethylaniline was prepared from *p*-nitrosodiethylaniline hydrochloride in the same way as the dimethyl compound,

[15] H. Rupe and J. Vśetečka: *Ann. d. Chem.*, ccci, p. 75, 1898.

except that the reduction was started in the cold and the double tin salt was filtered off after chilling, saturation of the reaction mixture with hydrochloric acid not being resorted to. The yield was the same as in the case of the dimethylamino compound. The substance is a pale yellow oil which boils at 139.5–40.5° at 9.5–10 mm. and melts below room temperature.

p-Chloroacetylaminodiethylaniline. This was prepared in the same way as the dimethylamino compound, and with approximately the same yield. Recrystallized from dilute alcohol, it forms faintly pink, delicate, lustrous needles, melting at 83–4° (corrected), and easily soluble in ether, chloroform, benzene, and acetone.

0.1993 gm. of substance (Kjeldahl) required 16.0 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{12}H_{17}ON_2Cl$: N = 11.65 per cent.
Found: N = 11.48 per cent.

p-Chloroacetylaminodiethylaniline and hexamethylenetetramine. The mechanical stimulus of filtration was sufficient to induce crystallization of the salt from the reaction mixture. The precipitation was made more complete by the addition of two volumes of dry acetone. The compound forms glistening micro-crystals melting with decomposition at 159–60° and dissolving readily in water and alcohol. The same precautions must be observed in the analysis as in the case of the dimethylamino analog.

0.2120 gm. of substance required 5.65 cc. $AgNO_3$ Solution I.
Calculated for $C_{18}H_{29}ON_6Cl$: Cl = 9.32 per cent.
Found: Cl = 9.38 per cent.

p-Nitrosodipropylaniline hydrochloride. 28 grams of dipropylaniline were dissolved in a mixture of 60 grams of concentrated hydrochloric acid and 125 grams of ice and water. A concentrated aqueous solution of 12.2 grams of sodium nitrite was then added, drop by drop, with stirring and chilling. After stirring for about half an hour, the nitroso hydrochloride was salted out and filtered off. A small portion was dissolved in hot absolute alcohol, bone-blacked, filtered, and precipitated by dry ether as a greenish yellow, micro-crystalline powder. The salt dissolves readily

in water, and, when heated rapidly, darkens and decomposes violently at about 160–5°. It is a powerful irritant to the mucous membrane.

0.4317 gm. of substance gave 0.2560 gm. AgCl.
Calculated for $C_{12}H_{18}ON_2{\cdot}HCl$: Cl = 14.61 per cent.
Found: Cl = 14.67 per cent.

p-Aminodipropylaniline. The crude, red-brown nitroso hydrochloride was reduced as in the case of the dimethylaniline analog. In this way, from 28 grams of dipropylaniline were obtained 15.5 grams of *p*-amino compound as a somewhat viscous, pale yellow oil of which most boiled at 155.5–6.5° at 6 mm. It does not solidify in a freezing mixture, is miscible in all proportions with absolute alcohol, and is slightly soluble in hot water.

0.1546 gm. of substance (Kjeldahl) required 15.85 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{12}H_{20}N_2$: N = 14.58 per cent.
Found: N = 14.37 per cent.

p-Chloroacetylaminodipropylaniline. The compound separated from the benzene layer of the reaction mixture after adding ligroin and letting stand over night in the ice box. Recrystallized from 95 per cent alcohol, it forms practically colorless prisms softening at 120° and melting at 121–1.5° (corrected). Yield: 14 grams from 15 grams of base.

0.2691 gm. of substance (Kjeldahl) required 20.13 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{14}H_{21}ON_2Cl$: N = 10.43 per cent.
Found: N = 10.48 per cent.

p-Chloroacetylaminodipropylaniline and hexamethylenetetramine. On one occasion the salt separated incompletely from the chloroform solution as an oil, in another preparation as a pasty mass. In both cases, about three volumes of dry acetone were added in order to complete the precipitation and induce crystallization. The compound forms minute platelets containing solvent of crystallization which is driven off *in vacuo* at the temperature of boiling alcohol. The salt then melts at 165–8° to a tar, and is difficultly soluble in water, easily in chloroform, absolute alcohol, and dilute acids.

0.2185 gm. of substance required 10.26 cc. $AgNO_3$ Solution II.[16]
Calculated for $C_{20}H_{33}ON_6Cl$: Cl = 8.68 per cent.
Found: Cl = 8.73 per cent.

p-Chloroacetylaminoethylbenzylaniline. p-Aminoethylbenzylaniline was prepared according to the method of Schultz, Rohde, and Bosch.[17] The chloroacetyl derivative separates from the benzene layer of the reaction mixture as an oil which rapidly solidifies. After letting stand over night in the ice box, the substance was filtered off and recrystallized from 95 per cent alcohol, with bone-blacking. It forms pale brownish microprisms melting at 106–8° to a brown liquid and is readily soluble in benzene. Yield: poor.

0.2128 gm. of substance (Kjeldahl) required 14.01 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{17}H_{19}ON_2Cl$: N = 9.26 per cent.
Found: N = 9.23 per cent.

p-Chloroacetylaminoethylbenzylaniline and hexamethylenetetramine. The salt separated incompletely from the chloroform solution as an oil, which was converted into crystalline form by the addition of dry acetone to the mixture. It forms glistening microplatelets melting with decomposition at 163–4° and is very difficultly soluble in water. An aqueous solution may be obtained, however, by adding a molecular equivalent of dilute hydrochloric acid.

0.2060 gm. of substance required 4.97 cc. $AgNO_3$ Solution I.
Calculated for $C_{23}H_{31}ON_6Cl$: Cl = 8.21 per cent.
Found: Cl = 8.49 per cent.

p-Chloroacetylaminoazobenzene. (See formula I.) The substance was filtered from the toluene layer of the reaction mixture and recrystallized from 95 per cent alcohol, with bone-blacking. Yield: about 50 per cent. Recrystallized from toluene, it forms sheaves of golden-yellow needles which soften at 155° and melt at 159.5° (corrected). The compound is soluble

[16] The pink color produced by the indicator may be temporarily destroyed by the addition of concentrated nitric acid.
[17] G. Schultz, G. Rohde, and E. Bosch: *Ann. d. chem.*, cccxxxiv, p. 262, 1904.

in hot chloroform and dissolves in sulphuric acid with a brown-orange color.

0.1409 gm. of substance gave 19.45 cc. N (752 mm. and 23.2°).
Calculated for $C_{14}H_{12}ON_3Cl$: N = 15.36 per cent.
Found: N = 15.30 per cent.

p-Chloroacetylaminoazobenzene and hexamethylenetetramine. The mechanical stimulus of filtering the deep red chloroform solution was sufficient to induce crystallization. The orange-yellow salt melts with decomposition at 153–4°. It is difficultly soluble in water, the solution dyeing silk a light yellow shade. The stability of the aqueous solution and the solubility are increased by the addition of a small amount of aqueous sodium carbonate. It turns red under sulphuric acid and dissolves with an orange color.

0.2756 gm. of substance required 6.63 cc. $AgNO_3$ Solution I.[18]
Calculated for $C_{20}H_{24}ON_7Cl$: Cl = 8.57 per cent.
Found: Cl = 8.47 per cent.

Chloroacetylaminoazotoluene (*o-tolueneazochloroacetyl-o-toluidine*). This was prepared from aminoazotoluene (from *o*-toluidine) in the same way and with the same yield as chloroacetylaminoazobenzene. Recrystallized first from toluene with the aid of a little ligroin, and then from absolute alcohol, it forms pale salmon-colored needles which soften at 168.5° and melt at 171–2.5° (corrected). The compound is difficultly soluble in the cold in the usual organic solvents, easily on warming, and gives a brown-red solution in sulphuric acid.

0.1417 gm. of substance (Kjeldahl) required 14.32 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{16}H_{16}ON_3Cl$: N = 13.93 per cent.
Found: N = 13.89 per cent.

Chloroacetylaminoazotoluene and hexamethylenetetramine. The orange-yellow salt melts at 167–9° to a red-brown liquid. It is somewhat soluble in chloroform and absolute alcohol and dissolves much more readily in water than the preceding unsubsti-

[18] Before titrating the substances of this type, dilute nitric acid was added to the hot solution. The mixture was cooled and the precipitate filtered off.

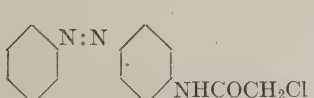
tuted homolog. It gives an orange color with sulphuric acid. The aqueous solution dyes silk a pale yellow shade.

0.2637 gm. of substance required 6.07 cc. $AgNO_3$ Solution I.
Calculated for $C_{22}H_{28}ON_7Cl$: Cl = 8.03 per cent.
Found: Cl = 8.10 per cent.

β-Naphthaleneazochloroacetyl-β-naphthylamine. The product obtained from 14.8 grams of β-naphthaleneazo-β-naphthylamine (aminoazo-β-naphthalene) as in the above cases was filtered off, washed with toluene, and recrystallized from acetone. Yield: 10.9 grams. Recrystallized first from toluene with the aid of a little ligroin and then from amyl alcohol, it melts with decomposition at 181.5–2.5° (corrected). The compound forms bright orange, hair-like needles, easily soluble in chloroform, difficultly in alcohol, and soluble in sulphuric acid with a deep violet color.

0.1387 gm. of substance gave 14.1 cc. N (757 mm. and 25.3°).
Calculated for $C_{22}H_{16}ON_3Cl$: N = 11.25 per cent.
Found: N = 11.24 per cent.

The hexamethylenetetramine addition product of the chloroacetyl derivative proved to be insoluble in water and could not be purified. Before this result was obtained we had already under way the preparation of other substances of this type, but owing to the unpromising properties of the above hexamethylenetetramine derivative this line of work was dropped. For purposes of record, however, we give below a description of the substances which had been prepared.



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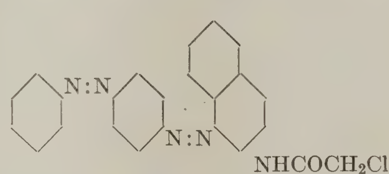

(II)

Benzeneazobenzeneazochloroacetyl-β-naphthylamine. Benzeneazobenzeneazo-β-naphthylamine (benzene-*p*-disazobenzene-β-naphthylamine) was chloroacetylated as in the previous cases. After cooling, the product (see formula II) was filtered off, washed with toluene, and recrystallized from this, with bone-blacking. Yield: about 60 per cent of the theory. The compound forms brick-

red micro-needles which darken above 188° and melt at 190° (corrected) with decomposition. It is very difficultly soluble in absolute alcohol, more readily in chloroform, and gives a red-brown solution with sulphuric acid.

0.1221 gm. of substance gave 18.4 cc. N (746 mm. and 22.4°).
Calculated for $C_{24}H_{18}ON_5Cl$: N = 16.38 per cent.
Found: N = 16.63 per cent.

o-Tolueneazo-o-tolueneazo-β-naphthylamine. 17.2 grams of aminoazotoluene (from *o*-toluidine) were stirred into 25 cc. of concentrated hydrochloric acid, after which the mixture was diluted with water, cooled with ice, and slowly diazotized with a little less than the theoretical amount of sodium nitrite. The solution of the diazonium salt was filtered into a well stirred suspension of β-naphthylamine hydrochloride obtained by rapidly chilling a solution of 10.9 grams of the amine in hot, dilute hydrochloric acid containing 9 cc. of concentrated acid. The mixture was turbined for one-half hour, filtered, and the solid washed successively with dilute hydrochloric acid, water, dilute aqueous ammonia, and water. The crude product was dissolved in hot acetone, bone-blacked, and crystallized by adding hot water and cooling. Yield: 13.8 grams. Recrystallized from toluene, the compound forms glistening, dark violet-brown leaflets which melt at 174–7° and dissolve in toluene and acetone with a deep red color, and in sulphuric acid with a deep blue-green color. In concentrated hydrochloric acid it dissolves with difficulty with a deep blue color.

0.1295 gm. of substance gave 21.25 cc. N (754 mm. and 23.8°).
Calculated for $C_{24}H_{21}N_5$: N = 18.45 per cent.
Found: N = 18.18 per cent.

o-Tolueneazo-o-tolueneazochloroacetyl-β-naphthylamine. The base was dissolved in warm toluene and chloroacetylated in the usual way after adding an excess of normal aqueous sodium hydroxide. Recrystallized twice from acetic acid and then from amyl alcohol, it melts to a tar at 122-6° with preliminary softening. It forms a dark brown micro-crystalline powder, gives a strong halogen test with copper wire, and yields deep red-brown solutions with chloroform, acetone, and benzene. The color in sulphuric acid is dark brown.

0.1220 gm. of substance gave 16.2 cc. N (755 mm. and 22°).
Calculated for $C_{26}H_{22}ON_5Cl$: N = 15.37 per cent.
Found: N = 14.87 per cent.

o-Tolueneazo-α-naphthylamine. Although this substance has been used by previous workers[19] and is easily prepared by diazotizing *o*-toluidine and coupling with α-naphthylamine in the usual manner, we were unable to find any description of it in the literature.[20] Recrystallized first from dilute alcohol and then ligroin, it forms rosettes of orange-red hairs melting at 97–7.5° (corrected) with slight preliminary softening. It is very soluble in the usual organic solvents, and gives deep red solutions in sulphuric and acetic acids. The hydrochloride is very difficultly soluble in water.

0.1243 gm. of substance gave 17.45 cc. N (758 mm. and 22°).
Calculated for $C_{17}H_{15}N_3$: N = 16.09 per cent.
Found: N = 15.78 per cent.

o-Tolueneazo-α-naphthaleneazo-β-naphthylamine. The above dye was diazotized, coupled, and basified as in the case of the corresponding aminoazotoluene derivative (see above). The yield was excellent. Recrystallized twice from amyl alcohol, the substance sinters above 140° and melts at about 173–8°. It forms a black-brown, micro-crystalline powder which is soluble in chloroform, acetone, and benzene with a purplish red color, and difficultly soluble in ether and absolute alcohol. It gives an intense blue color with sulphuric acid, dissolves with difficulty in dilute hydrochloric acid with a faint purple color, and is insoluble in dilute nitric and sulphuric acids. The fact that the compound burned with difficulty probably explains the low result on analysis.

0.1360 gm. of substance gave 19.45 cc. N (763 mm. and 21.3°).
Calculated for $C_{27}H_{21}N_5$: N = 16.87 per cent.
Found: N = 16.23 per cent.

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[19] Compare R. Nietzki and R. Zehntner: *Ber. d. deutsch. chem. Gesellsch.*, xxvi, p. 145, 1893.

[20] After this paper had been sent to press we found a description of this substance by L. Casale and M.Casale-Sacchi (*Gazz. chim. ital.*, xliv, pt. ii, p. 395, 1914), who give the melting point as 95°.

As there seemed to be little hope for the preparation of water-soluble hexamethylenetetramine dyes from intermediates containing benzene and naphthalene radicals with neutral substituents only, it was decided to experiment with dyes containing salt-forming groups. The series of dyes described below was developed with this in view.

p-Acetaminobenzeneazodimethylaniline. This compound was prepared by Meldola[21] by the reduction and acetylation of the corresponding nitro compound. As this method did not give good results in our hands, the following procedure was used: The diazonium solution from 19.8 grams of *p*-aminoacetanilide was slowly run into an alcoholic solution of 32.3 grams of dimethylaniline, with cooling and vigorous stirring. The separation of the orange-brown dye was completed by the addition of sodium acetate, after which the product was filtered off and washed with 50 per cent alcohol. Yield: 31.4 grams, melting at 223–7°. Recrystallized from 90 per cent alcohol, it melts at 226–7° and not at 217°, as stated by Meldola.

p-Aminobenzeneazodimethylaniline. The above acetyl derivative was boiled for thirty-five minutes with about eleven parts of sulphuric acid (1:4), the mixture gradually assuming a thinner consistency as the hydrolysis proceeded. The solution was cooled and, with continued cooling, made slightly alkaline with aqueous ammonia. The red-brown, bulky precipitate was filtered off, washed with water and 50 per cent alcohol, and dried. Yield: 22.5 grams.

p-Chloroacetylaminobenzeneazodimethylaniline. 7.5 grams of amino compound were dissolved as well as possible in about 100 cc. of warm benzene and chloroacetylated in the usual manner with chloroacetyl chloride and normal sodium hydroxide solution. The dark brown precipitate was filtered off, washed well with toluene, water, and 50 per cent alcohol, and dried. Yield: 8.5 grams. Recrystallized from boiling isoamyl alcohol, in which the substance is difficultly soluble, it forms glistening, chocolate-brown micro-plates, soluble in chloroform and acetone. The solution in dilute mineral acids dyes silk a brownish yellow shade. When rapidly heated, the compound sinters at about 190° and melts at about 205°.

[21] R. Meldola: *Jour. Chem. Soc.*, xlv, p. 108, 1884.

0.2097 gm. of substance (Kjeldahl) required 26.45 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{16}H_{17}ON_4Cl$: N = 17.71 per cent.
Found: N = 17.68 per cent.

p-Chloroacetylaminobenzeneazodimethylaniline and hexamethylenetetramine. The brown salt was washed well with chloroform and boiled with dry acetone. It melts at 167–8.5° and is difficultly soluble in water, more easily on adding a dilute mineral acid, the color of the solution changing from orange to deep purple. It dyes silk a brown-orange color.

0.1189 gm. of substance gave 25.4 cc. N (762 mm. and 20.5°).[22]
0.3075 gm. of substance gave 0.1001 gm. AgCl.
Calculated for $C_{22}H_{29}ON_8Cl$: N = 24.53 per cent; Cl = 7.76 per cent.
Found: N = 24.33 per cent; Cl = 8.05 per cent.

p-Acetaminobenzeneazodiethylaniline. This substance was prepared in the same way as the dimethyl homolog. Yield of crude product: 35 grams. 5 grams of this, recrystallized from 85 per cent alcohol, yielded 4.8 grams of glistening, orange-brown plates which form an orange-red powder when pulverized and soften at 186° to a semicrystalline mass which becomes entirely fluid at 192°. The solution in dilute hydrochloric acid dyes silk a brownish yellow shade.

0.1536 gm. of substance (Kjeldahl) required 19.77 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{18}H_{22}ON_4$: N = 18.06 per cent.
Found: N = 18.05 per cent.

p-Aminobenzeneazodiethylaniline. 30 grams of acetamino derivative were saponified as in the case of the dimethyl compound. The partly resinous product solidified on standing, and was ground up in a mortar and dried. Yield: 23.2 grams. Recrystallized first from benzene with the aid of an equal volume of ligroin, and then twice from 85 per cent alcohol, it forms dark brown crystals with a blue-black luster. These melt at 149–50° (corrected) with preliminary softening.

0.1529 gm. of substance (Kjeldahl) required 22.63 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{16}H_{20}N_4$: N = 20.89 per cent.
Found: N = 20.74 per cent.

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[22] Quantitative results were obtained only when the substance was mixed with lead chromate.

p-Chloroacetylaminobenzeneazodiethylaniline. 15 grams of amino compound were partially dissolved in warm toluene and chloroacetylated as in the case of the dimethyl homolog. Precipitation of the chloroacetyl derivative was assisted by adding an equal volume of ligroin to the toluene solution before cooling. The product was combined with a small amount obtained by extracting the toluene-ligroin solution with 5 per cent sulphuric acid and making alkaline, and then recrystallized by bone-blacking in benzene and adding ligroin to the hot, filtered solution. Yield: 9.6 grams of chocolate-brown crystals, which sinter above 155° and melt at 160.5–2°. The solution in dilute mineral acids dyes silk a brown-orange shade.

0.1490 gm. of substance (Kjeldahl) required 17.27 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{18}H_{21}ON_4Cl$: N = 16.26 per cent.
Found: N = 16.25 per cent.

p-Chloroacetylaminobenzeneazodiethylaniline and hexamethylenetetramine. Precipitation of the brown-orange salt from the chloroform solution was completed by adding dry acetone and cooling in the ice box. The product melts at 172–3° with preliminary sintering, is somewhat hygroscopic, and is easily soluble in water, yielding a brown-orange solution which dyes silk the same color.

0.2787 gm. of substance gave 0.0868 gm. AgCl.
Calculated for $C_{24}H_{33}ON_8Cl$: Cl = 7.32 per cent.
Found: Cl = 7.71 per cent.

p-Acetaminobenzeneazodipropylaniline. 15 grams of *p*-aminoacetanilide were diazotized and the solution was added, with stirring, to a cold solution of 17.7 grams of dipropylaniline in about 300 cc. of 85 per cent alcohol containing 20 grams of sodium acetate. During the coupling process, which took place rapidly, a little 95 per cent alcohol was added in order to prevent the separation of unchanged dipropylaniline. After fifteen minutes' stirring, the mixture was allowed to stand for one hour, whereupon the precipitation of the dye was completed by the addition of water. Yield: 26.4 grams. Recrystallized first from 95 per cent alcohol and then from toluene, the compound forms small, orange needles which, when rapidly heated, melt

at 178° (corrected). In dilute hydrochloric acid it dyes silk a brownish yellow shade.

0.1227 gm. of substance gave 18.25 cc. N (760 mm. and 25.1°).
Calculated for $C_{20}H_{26}ON_4$: N = 16.56 per cent.
Found: N = 16.50 per cent.

p-Chloroacetylaminobenzeneazodipropylaniline. The acetamino compound (23 grams) was hydrolyzed as in the previous examples, but as difficulty was experienced in obtaining the amino derivative in a crystalline state, the crude, resinous product was dissolved in benzene and chloroacetylated in the usual manner with 100 cc. of two-normal sodium hydroxide solution and 5 cc. of chloroacetyl chloride. The benzene layer was evaporated to small bulk in the cold by means of a current of air, yielding, in all, 9.4 grams of red-brown micro-platelets. Recrystallized again in the same manner, the substance forms orange-brown aggregates of micro-crystals melting at 112–4° (corrected) with preliminary softening and darkening. It is readily soluble in benzene and chloroform, less so in absolute alcohol.

0.1581 gm. of substance (Kjeldahl) required 16.67 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{20}H_{25}ON_4Cl$: N = 15.03 per cent.
Found: N = 14.78 per cent.

p-Chloroacetylaminobenzeneazodipropylaniline and hexamethylenetetramine. The solid cake formed on boiling the components for forty minutes in dry chloroform solution was disintegrated, treated with about two volumes of dry acetone, and filtered off. The salt was then ground up in a mortar with dry acetone and washed well with this, being obtained finally in the form of orange-yellow micro-platelets which soften and sinter above 100° and melt completely at 180–1°. It is somewhat less soluble in water than the diethyl homolog and dyes silk a brown-orange shade.

0.3125 gm. of substance required 6.47 cc. $AgNO_3$ Solution I.[23]
Calculated for $C_{26}H_{37}ON_8Cl$: Cl = 6.92 per cent.
Found: Cl = 7.29 per cent.

[23] After dissolving the substance in fairly strong nitric acid and adding an excess of the silver nitrate solution, the mixture was heated at 100° until most of the dye was destroyed. The precipitate was filtered off and the silver determined gravimetrically in the filtrate.

p-Acetaminobenzeneazoethylbenzylaniline. The diazonium solution from 20 grams of *p*-aminoacetanilide was slowly run into a well stirred solution of 28 grams of ethylbenzylaniline in about 500 cc. of absolute alcohol containing 15 grams of sodium bicarbonate in suspension. As coupling took place very slowly, about 5 cc. of concentrated aqueous ammonia were added and the stirring continued about three hours. Aqueous ammonia was then added in slight excess, followed by about two volumes of water. This precipitated a dark colored oil which solidified almost completely on standing several days. The product was filtered off and dried *in vacuo* over sulphuric acid, after which it was extracted with cold ether and washed well with this. The substance was thus obtained in a yield of 23.7 grams as a chocolate-brown powder. Recrystallized from 85 per cent alcohol, it forms minute brown rhombs with a violet reflex. On drying *in vacuo* at the temperature of boiling alcohol, water is given off and the color changes to red-brown.

0.8248 gm. of substance lost 0.0230 gm.
Calculated for $C_{23}H_{24}ON_4 \cdot \frac{1}{2}H_2O$: H_2O = 2.36 per cent.
Found: H_2O = 2.79 per cent.

0.1819 gm. of dried substance (Kjeldahl) required 19.37 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{23}H_{24}ON_4$: N = 15.05 per cent.
Found: N = 14.93 per cent.

Recrystallized from benzene, the compound forms yellow-brown spears with a violet luster which melt at 148–50° (corrected) with slight preliminary reddening and softening. These form an ochre powder when pulverized, and, in very dilute hydrochloric acid solution, dye silk a lemon-yellow shade.

The *amino derivative*, obtained by hydrolysis of the acetyl compound by boiling for one-half hour with ten parts of sulphuric acid (1:4), is best isolated by allowing the cooled solution to run slowly into well chilled and rapidly stirred dilute ammonia. In this way the base separates in the form of brown, amorphous flocks which coalesce to a thick resin on filtering. The crude product was taken up in benzene and used for the preparation of the chloroacetyl derivative.

p-Chloroacetylaminobenzeneazoethylbenzylaniline. 150 cc. of normal sodium hydroxide solution were added to the above ben-

zene solution and the mixture was thereupon treated with 6 cc. of chloroacetyl chloride, added in small portions, with vigorous shaking. The benzene layer was evaporated to small bulk *in vacuo* in the cold, depositing brown crystalline crusts which were recrystallized from toluene. Yield: 6.5 grams. The compound melts at 140–1.5° (corrected) and is easily soluble in acetone and chloroform, difficultly in cold alcohol and toluene. It dissolves in acetic acid with a deep red color. The solution in dilute hydrochloric acid dyes silk a brownish yellow shade.

0.1367 gm. of substance gave 16.7 cc. N (764 mm. and 23.7°).
Calculated for $C_{23}H_{23}ON_4Cl$: N = 13.78 per cent.
Found: N = 13.73 per cent.

p-Chloroacetylaminobenzeneazoethylbenzylaniline and hexamethylenetetramine. After about twenty minutes' boiling the chloroform solution set to a solid cake. This was broken up, boiled ten minutes longer with a little more dry chloroform, filtered, and washed cautiously with dry chloroform in order to remove the brown color. Excessive amounts of solvent must be avoided, as the salt is somewhat soluble in chloroform. After grinding up with dry acetone, the residue forms ochreous micro-plates which gradually sinter above 110° and melt to a tar at 140°. The salt dissolves in water, forming an orange solution which dyes silk an orange-red color. It is also soluble in alcohol.

0.2588 gm. of substance required 4.53 cc. $AgNO_3$ Solution I.[24]
Calculated for $C_{29}H_{35}ON_8Cl$: Cl = 6.48 per cent.
Found: Cl = 6.16 per cent.

All the above dialkylamino dyes dissolve in sulphuric acid with brownish orange colors and in dilute mineral acids with the formation of purple solutions.

m-Bromodiethylaniline. Twenty grams of *m*-bromoaniline, 56 grams of ethyl iodide, 12 grams of sodium hydroxide, and about 50 cc. of absolute alcohol were heated at 100° in a pressure bottle for eight hours, with occasional shaking. Water was added, the precipitated oil taken up in ether, and the bases were extracted

[24] The solution was warmed with nitric acid until pale yellow, after which an excess of the silver nitrate solution was added, the tar and silver chloride filtered off, and the titration completed as usual.

with 10 per cent sulphuric acid. They were then precipitated with sodium hydroxide, taken up with ether, dried, and the solution evaporated to small bulk. The residue was then warmed for three hours on the water bath with acetic anhydride in order to acetylate the primary and secondary amines. Ether was then added and the tertiary amine extracted by shaking with 10 per cent sulphuric acid. The base was precipitated with sodium hydroxide, taken up in ether, and the solution dried over potassium hydroxide and evaporated to small bulk. On fractionating *in vacuo* the substance was obtained in a yield of 10.1 grams as a highly refracting, colorless liquid boiling at 139.5–142° (corrected) at 9–10 mm. It is heavier than water, has a slight aromatic odor, and solidifies in a freezing mixture, melting again at a low temperature.

0.1373 gm. of substance (Kjeldahl) required 6.22 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{10}H_{14}NBr$: N = 6.14 per cent.
Found: N = 6.35 per cent.

p-Acetaminobenzeneazo-2′-bromo-4′-diethylaminobenzene. The dye was prepared similarly to the ethylbenzylaniline analog. A small amount of tar was obtained, which solidified on long keeping. Recrystallized twice from absolute alcohol, it forms minute, glistening rhombs which sinter and redden above 160° and melt at 163.5–4° (corrected). It readily forms highly supersaturated solutions and is very soluble in chloroform, less so in benzene, acetone, hot alcohol, and hot amyl alcohol. The dark violet solution in dilute hydrochloric acid dyes silk a deep yellow shade. The substance gives a strong Beilstein test and dissolves in sulphuric acid with a red-brown color.

0.1510 gm. of substance gave 19.3 cc. N (771 mm. and 22°).
Calculated for $C_{18}H_{21}ON_4Br$: N = 14.39 per cent.
Found: N = 14.62 per cent.

Benzeneazo-2′-chloroacetylamino-4′-dimethylaminobenzene. This substance of the chrysoidine type was prepared by diazotizing and coupling 2.1 grams of aniline with a solution of 5 grams of *m*-chloroacetylaminodimethylaniline (page 113) in one molecular equivalent of 10 per cent hydrochloric acid, following the directions for the preparation of chrysoidine as given in Möhlau

and Bucherer's *Farbenchemisches Praktikum*, p. 132 (Leipsic, 1908), except that the heating was omitted. Recrystallization of the crude product from absolute alcohol yielded 2.9 grams of scarlet tablets with a faint violet luster. The compound melts at 109.5–10° (corrected) with slight preliminary softening, is readily soluble in chloroform and benzene, and dissolves in sulphuric acid with a bright orange color. It is difficultly soluble in cold, dilute hydrochloric acid, the solution dyeing silk an orange shade.

0.1430 gm. of substance (Kjeldahl) required 17.9 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{16}H_{17}ON_4Cl$: N = 17.71 per cent.
Found: N = 17.53 per cent.

The hexamethylenetetraminium salt was obtained in poor yield and in a very impure condition.

4-Nitrobenzeneazo-2′-chloroacetylamino-4′-dimethylaminobenzene. The diazonium solution obtained from 3.25 grams of *p*-nitraniline was diluted to about 600 cc. To the well stirred solution were added, in small portions, 5 grams of finely powdered *m*-chloroacetylaminodimethylaniline, the solution becoming a dark violet-brown. After one-half hour's stirring, normal sodium acetate solution was added, drop by drop, until the reaction became neutral to Congo Red paper. After standing over night in the ice box, the violet-brown precipitate was filtered off, dried, and recrystallized from hot chloroform, in which it is very difficultly soluble. The substance was obtained in this way in the form of dark violet-brown, voluminous, triboëlectric masses of microcrystals. When rapidly heated it melts at 220°. It is difficultly soluble in the usual solvents, excepting hot nitrobenzene, from which it separates in dark crystals with a metallic green reflex. It gives a bright red color with sulphuric acid and dissolves with difficulty in dilute hydrochloric acid with a brownish violet color.

0.1642 gm. of substance (Kjeldahl) required 22.45 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{16}H_{16}O_3N_5Cl$: N = 19.31 per cent.
Found: N = 19.16 per cent.

Most of the substance was recovered unchanged after heating in a pressure bottle at 100° with hexamethylenetetramine in dry chloroform for several hours.

The following two substances were encountered in the course of experiments which were not carried to completion:

p-Diethylaminobenzeneazo-β-naphthylamine. 18 grams of *p*-aminodiethylaniline were diazotized in the usual manner, and the solution was poured slowly into a well stirred solution of 15.5 grams of β-naphthylamine in about 150 cc. of warm 90 per cent alcohol. A deep red color developed at once. After about fifteen minutes, sodium acetate was added in excess, precipitating a gummy mass which solidified on standing over night. 9.3 grams were obtained after recrystallization from 95 per cent alcohol. After another recrystallization, the compound forms glistening, maroon plates melting at 117–20° with preliminary softening. When pulverized, it forms a brick-red powder, readily soluble in benzene and chloroform and dissolves in dilute acids to form deep red solutions which dye silk a brown-orange color.

0.1208 gm. of substance gave 19.65 cc. N (763 mm. and 23.5°).
Calculated for $C_{20}H_{22}N_4$: N = 17.60 per cent.
Found: N = 18.27 per cent.

p-Diethylaminobenzeneazochloroacetyl-α-naphthylamine. 11 grams of *p*-aminodiethylaniline were diazotized and the solution was slowly added to a well stirred solution of 10 grams of α-naphthylamine in warm 90 per cent alcohol. Stirring was continued for one hour, after which sodium acetate was added, precipitating a resinous mass which eventually became of such consistency that it could be filtered off and dried. As the amine could not be obtained in a crystalline state, it was dissolved in benzene and treated with one molecular equivalent of chloroacetic anhydride, the solution being eventually warmed. On cooling, a gum separated. This solidified on long standing, after which it was pulverized, washed well with dilute alkali and water, dried, and recrystallized from amyl alcohol, yielding 7.8 grams of chocolate-brown micro-crystals. These darken and soften above 120° and melt at 145° to a thick tar.

0.1716 gm. of substance (Kjeldahl) required 17.35 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{22}H_{23}ON_4Cl$: N = 14.19 per cent.
Found: N = 14.16 per cent.

The hexamethylenetetraminium salt was obtained only in an impure condition.

o-Chloroacetylaminophenol was obtained in practically quan-

titative yield by dissolving 4 grams of *o*-aminophenol in boiling benzene, adding 7 grams of chloroacetic anhydride, and boiling the resulting solution for fifteen minutes. The substance separated on cooling as a thick mass of needles and conformed to the description given by Aschan.[25]

o-Chloroacetylaminophenol and hexamethylenetetramine. The components were boiled for fifteen minutes in dry chloroform, the clear solution first obtained being concentrated to small bulk at the same time. A précipitation of caseous material occurred, which rapidly became crystalline. The product was filtered off, ground up with dry chloroform, washed with dry acetone, and finally boiled one-half hour with dry acetone in order to remove a small amount of material insoluble in water. The salt melts with decomposition at 155°, dissolves in absolute alcohol, and is readily soluble in water, the solution giving a purple color with ferric chloride.

0.2676 gm. of substance gave 0.1194 gm. AgCl.
Calculated for $C_{14}H_{20}O_2N_5Cl$: Cl = 10.89 per cent.
Found: Cl = 11.04 per cent.

o-Chloroacetylaminophenyl benzoate. 3 grams of benzoyl chloride were carefully added to a well chilled solution of 4 grams of *o*-chloroacetylaminophenol in 20 grams of pyridine. After fifteen minutes, the mixture was poured into water, precipitating an oil which solidified on scratching. The yield was practically quantitative. Recrystallized first from absolute, then 85 per cent alcohol, the benzoate forms silky needles melting at 107.5–8.5° (corrected). It is very soluble in chloroform and benzene, rather difficultly in dry ether.

0.2041 gm. of substance (Kjeldahl) required 7.1 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{15}H_{12}O_3NCl$: N = 4.84 per cent.
Found: N = 4.87 per cent.

o-Chloroacetylaminophenyl benzoate and hexamethylenetetramine. The clear solution obtained on boiling the components in dry chloroform was evaporated to small bulk and treated with dry acetone. The precipitated salt, which retained chloroform of crystallization, even on drying to constant weight *in vacuo* at

[25] O. Aschan: *Ber. d. deutsch. chem. Gesellsch.*, xx, p. 1524, 1887.

the temperature of boiling alcohol over sulphuric acid, was boiled with dry acetone. As a result, acetone of crystallization was substituted for the chloroform and was held with equal tenacity. The presence of the acetone was proven by the iodoform test.

0.2035 gm. of substance required 8.12 cc. $AgNO_3$ Solution II.
Calculated for $C_{21}H_{24}O_3N_5Cl \cdot CH_3COCH_3$: Cl = 7.27 per cent.
Found: Cl = 7.42 per cent.

o-Chloroacetylaminophenyl p-nitrobenzoate. 2.3 grams of *o*-chloroacetylaminophenol were dissolved in 15 grams of pyridine, cooled in a freezing mixture, and treated with a solution of 2.3 grams of *p*-nitrobenzoyl chloride in a little chloroform. After ten minutes at room temperature, the mixture was poured into dilute sulphuric acid and the chloroform removed by a current of air. The ester was filtered off and dried. Yield: practically quantitative. Recrystallized first from absolute alcohol, then toluene, it forms delicate, interlaced needles which soften at about 160° and melt at 163.5–4° (corrected). It is difficultly soluble in cold absolute alcohol, benzene, and toluene, readily in chloroform.

0.1450 gm. of substance (Kjeldahl) required 8.45 cc. $\frac{N}{10}$ HCl.
0.1685 gm. of substance gave 0.0713 gm. AgCl.[7]
Calculated for $C_{15}H_{11}O_5N_2Cl$: N = 8.37 per cent; Cl = 10.59 per cent.
Found: N = 8.16 per cent; Cl = 10.47 per cent.

o-Chloroacetylaminophenyl p-nitrobenzoate and hexamethylenetetramine. To the clear solution obtained by boiling the components in a small volume of dry chloroform were added several volumes of dry acetone. After crystallization had been induced by scratching, the mixture was allowed to stand over night. The salt forms pale yellow plates which darken at about 166° and melt at 171–2°.

0.1798 gm. substance required 4.05 cc. $AgNO_3$ Solution I.
Calculated for $C_{21}H_{23}O_5N_6Cl$: Cl = 7.46 per cent.
Found: Cl = 7.92 per cent.

m-Chloroacetylaminophenol. 8.5 grams of chloroacetic anhydride were added to a solution of 5 grams of *m*-aminophenol in boiling benzene. The solution was boiled for three-quarters of an hour and filtered from a small amount of tar which had

deposited. On cooling, a tar first separated, followed by a crystalline deposit which was increased by adding ligroin. Recrystallized from water, the compound forms almost colorless, glistening plates melting at 134.5–6.° (corrected). Yield: 3.8 grams. It is easily soluble in dilute ammonia and sodium carbonate solutions and dissolves with difficulty in chloroform.

0.2157 gm. of substance (Kjeldahl) required 11.4 cc. $\frac{N}{10}$ HCl.
Calculated for $C_8H_8O_2NCl$: N = 7.55 per cent.
Found: N = 7.40 per cent.

m-Chloroacetylaminophenol and hexamethylenetetramine. When a solution of the components in dry chloroform was boiled, a gummy mass soon separated. After about fifteen minutes' boiling, dry acetone was added, causing partial crystallization. After several days, during which most of the product had crystallized, the salt was filtered off, boiled about fifteen minutes with a little dry chloroform, and finally with dry acetone. When dried to constant weight *in vacuo* over calcium chloride and paraffin at room temperature, the salt retained acetone of crystallization. This was shown by the analysis and by the strong iodoform test given by the distillate from the aqueous solution. The salt decomposes at 168° with preliminary darkening. It is easily soluble in water, the solution giving a violet color with ferric chloride.

0.2657 gm. of substance gave 0.1067 gm. AgCl.
Calculated for $C_{14}H_{20}O_2N_5Cl \cdot \frac{1}{2}CH_3COCH_3$: Cl = 10.00 per cent.
Found: Cl = 9.94 per cent.

Benzeneazo-m-chloroacetylaminophenol. A solution of 1.5 grams of aniline, diazotized in the usual way, was added to a well stirred solution of 2.8 grams of *m*-chloroacetylaminophenol in about 400 cc. of ice water containing 2.4 grams of sodium hydroxide. After fifteen minutes, the dye was salted out from the dark red-brown solution, ground up to a paste with ice water, stirred well, and treated with a slight excess of dilute sulphuric acid. The product was filtered off, washed well with cold water, and recrystallized twice from absolute alcohol, forming minute, golden, interlaced needles. Yield: 3 grams. The compound melts at 200° (corrected) with decomposition. It is soluble in sulphuric acid with

a brown-orange color and gives a yellow solution with dilute sodium hydroxide which dyes silk a very pale yellow shade.

0.1095 gm. of substance gave 14.0 cc. N (765 mm. and 23°).
Calculated for $C_{14}H_{12}O_2N_3Cl$: N = 14.51 per cent.
Found: N = 14.44 per cent.

Benzeneazo-m-chloroacetylaminophenol and hexamethylenetetramine. As the insolubility of the dye in chloroform prevented the use of this solvent, the components were heated in a sealed tube with dry acetone at 100° for about one and one-half hours. The crude product was ground up and boiled out with dry acetone. The yellow salt thus obtained decomposes from 202 to 230°. It is insoluble in water, but readily gives a clear, orange solution with a little dilute sodium hydroxide, dyeing silk a pale yellow shade.

0.2835 gm. of substance (Carius) gave 0.0895 gm. AgCl.
Calculated for $C_{20}H_{24}O_2N_7Cl$: Cl = 8.25 per cent.
Found: Cl = 7.81 per cent.

p-Chloroacetylaminophenyl chloroacetate. This substance was obtained in an attempt to prepare *p*-chloroacetylaminophenol by the pyridine method. On pouring the reaction mixture into iced 25 per cent sulphuric acid, only a small amount of precipitate was obtained. This was washed well with water and recrystallized first from absolute alcohol, then toluene, forming practically colorless spears softening at 168° and melting at 170–1° (corrected). The substance is insoluble in dilute acids or alkalies and is saponified by warming with dilute sodium hydroxide.

0.2062 gm. of substance (Kjeldahl) required 7.90 cc. $\frac{N}{10}$ HCl.
0.2769 gm. of substance gave 0.3026 gm. AgCl.[7]
Calculated for $C_{10}H_9O_3NCl_2$: N = 5.35 per cent; Cl = 27.06 per cent.
Found: N = 5.37 per cent; Cl = 27.04 per cent.

Chloroacetyl-*o*-anisidine. In spite of the fact that this substance was used by Majert[26] and Frerichs[6] we were unable to find a description of it in the literature. We prepared it in the usual manner from *o*-anisidine in benzene solution, aqueous sodium hy-

[26] D. R. P. 59121; *Friedlaenders Fortschr. d. Teerfarbenfabrikation*, iii, p. 915, 1890–94.

droxide, and chloroacetyl chloride. The benzene layer was shaken out successively with dilute hydrochloric acid, water, sodium carbonate solution, and water, and finally dried and evaporated to small bulk *in vacuo.* The residue crystallized on chilling and rubbing, and, after two recrystallizations from absolute alcohol with the aid of a freezing mixture, formed flat needles melting at 48–8.5° (corrected) with slight preliminary softening. It is readily soluble at room temperature in the usual organic solvents except ligroin, and gives a faint pink color with sulphuric acid.

0.3329 gm. of substance (Kjeldahl) required 16.8 cc. $\frac{N}{10}$ HCl.
Calculated for $C_9H_{10}O_2NCl$: N = 7.02 per cent.
Found: N = 7.07 per cent.

Chloroacetyl-o-anisidine and hexamethylenetetramine. The solution obtained by boiling the components in dry chloroform was evaporated to small bulk. The salt separated out in almost quantitative yield after the addition of dry ether. It melts at 151–3°, is readily soluble in water, and dissolves in sulphuric acid with a violet-rose color.

0.2247 gm. of substance required 6.65 cc. $AgNO_3$ Solution I.
Calculated for $C_{15}H_{22}O_2N_5Cl$: Cl = 10.44 per cent.
Found: Cl = 10.42 per cent.

α-Iodopropionyl-o-anisidine: 11.5 grams of α-iodopropionyl chloride[27] were added, drop by drop, to a solution of 16 grams of *o*-anisidine in alcohol-free ether, with cooling and stirring. After warming for a few minutes, the mixture was allowed to stand in the ice box for several days. The *o*-anisidine hydrochloride was filtered off and the filtrate shaken out successively wth dilute hydrochloric acid containing a little sodium bisulphite, with water, dilute sodium carbonate solution, and water. The ether solution was then dried over calcium chloride, evaporated to dryness, and the residue recrystallized from ligroin, forming pale cream-colored, felted needles. Yield: 14.9 grams. It is readily soluble in the usual organic solvents. Recrystallized from dry, alcohol-free ether, it forms delicate needles melting at 95–6.5° (corrected) with slight preliminary softening.

[27] E. Abderhalden and M. Guggenheim: *Ber. d. deutsch. chem. Gesellsch.*, xli, p. 2855, 1908.

0.3119 gm. of substance (Kjeldahl) required 10.2 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{10}H_{12}O_2NI$: N = 4.59 per cent.
Found: N = 4.58 per cent.

When heated with hexamethylenetetramine in dry chloroform or dry acetone, the substance yielded, in addition to unchanged materials, only hexamethylenetetraminium iodide.

β-Iodopropionyl chloride. This was prepared essentially according to Abderhalden and Gressel,[28] who, however, did not describe their product. The chloride boils at 80.5–1.5° at 15 mm., and, after shaking with mercury to remove a small amount of dissolved iodine, forms a practically colorless, heavy, strong smelling oil.

0.1702 gm. of substance required 7.88 cc. $AgNO_3$ Solution I.[29]
Calculated for C_3H_4OClI: Cl = 16.24 per cent.
Found: Cl = 16.30 per cent.

β-Iodopropionyl-o-anisidine. This was prepared in the same way as the α-iodo analog. The reaction mixture was well shaken and allowed to stand about two hours before filtering off the hydrochloride of the amine. It was found necessary to use a large volume of ether in order to prevent the acyl derivative from crystallizing out with the hydrochloride. Yield: 19.4 grams from 19 grams of *o*-anisidine and 15 grams of β-iodopropionyl chloride, after recrystallization from dilute alcohol. Recrystallized first from methyl alcohol and then benzene, the compound forms colorless, irregular plates which soften at 88° and melt at 89–91° (corrected). It is much less soluble than the α-isomer.

0.3113 gm. of substance (Kjeldahl) required 10.1 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{10}H_{12}O_2NI$: N = 4.59 per cent.
Found: N = 4.54 per cent.

β-Iodopropionyl-o-anisidine and hexamethylenetetramine. A solution of equimolecular amounts of the components in dry chloroform was heated to the boiling point, whereupon the salt started to separate in thin, glistening, micro-platelets. After boiling

[28] E. Abderhalden and E. Gressel: *Ztschr. f. physiol. Chem.*, lxxiv, p. 476, 1911.

[29] Hydrolysis with cold, dilute, aqueous ammonia.

for fifteen minutes, the salt was filtered off. Additional quantities were obtained by longer boiling of the filtrate. The salt melts at 172–3° and is very difficultly soluble in water.

0.2237 gm. of substance required 5.15 cc. $AgNO_3$ Solution I.
Calculated for $C_{16}H_{24}O_2N_5I$: I = 28.51 per cent.
Found: I = 28.98 per cent.

Chloroacetyl-ω-o-anisidinoacetophenone. 7.5 grams of phenacyl-*o*-anisidine[30] were dissolved in benzene and 2.9 cc. of chloroacetyl chloride slowly added, with cooling and shaking. The mixture, which had turned green and contained a curdy precipitate, was warmed about three hours on the water bath, hydrochloric acid being evolved. After cooling, the solution was filtered and treated with several volumes of ligroin. An oil precipitated, which soon solidified and was recrystallized from dilute alcohol, with bone-blacking. In this way were obtained 7.7 grams of prisms. Recrystallized from methyl alcohol, the substance forms prismatic needles melting at 101.5–2.5° (corrected). It is soluble in benzene, difficultly in cold methyl alcohol and ether.

0.3305 gm. of substance (Kjeldahl) required 10.15 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{17}H_{16}O_3NCl$: N = 4.42 per cent.
Found: N = 4.30 per cent.

Chloroacetyl-ω-o-anisidinoacetophenone and hexamethylenetetramine. After the chloroform solution of the components had been boiled for about twelve hours, the small amount of precipitate was filtered off and the solution allowed to stand. The salt slowly separated in poor yield in the form of colorless micro-prisms. It melts at 145° with decomposition and is readily soluble in water and alcohol. The solution in sulphuric acid has a pale brownish yellow color.

0.2501 gm. of substance required 5.73 cc. $AgNO_3$ Solution I.
Calculated for $C_{23}H_{28}O_3N_5Cl$: Cl = 7.75 per cent.
Found: Cl = 8.06 per cent.

Chloroacetyl-*p*-anisidine. This substance was also used by Majert[26] and Frerichs,[6] but was not described. It was prepared by us in the usual way from *p*-anisidine in benzene solution, aqueous sodium hydroxide, and chloroacetyl chloride. Ligroin

[30] M. Busch and G. Hefele: *Jour. f. prakt. Chem.*, lxxxiii, p. 442, 1911.

was added to the benzene layer and the solution allowed to stand in the cold over night. The deposited substance was then recrystallized twice from absolute alcohol, forming glistening, transparent prisms melting at 121.5–2.5° (corrected) with preliminary softening. It is difficultly soluble in the cold in benzene, fairly readily in ether, chloroform, and alcohol. It gives no color in sulphuric acid.

0.3091 gm. of substance (Kjeldahl) required 15.02 cc. $\frac{N}{10}$ HCl.
Calculated for $C_9H_{10}O_2NCl$: N = 7.02 per cent.
Found: N = 6.81 per cent.

Chloroacetyl-p-anisidine and hexamethylenetetramine. The salt melts at 164–7° with decomposition. It dissolves readily in water and gives a pale brownish yellow solution with sulphuric acid.

0.1971 gm. of substance required 10.73 cc. $AgNO_3$ Solution II.
Calculated for $C_{15}H_{22}O_2N_5Cl$: Cl = 10.44 per cent.
Found: Cl = 10.13 per cent.

o-Chloroacetylaminobenzyl alcohol. 12.3 grams of *o*-aminobenzyl alcohol were dissolved in dry, alcohol-free ether, well cooled, and treated with a solution of 5.5 grams of chloroacetyl chloride in dry benzene. The mixture was treated with water to dissolve the precipitated hydrochloride, dried, and concentrated to small bulk. The residue, which crystallized on cooling, was recrystallized from a small volume of benzene with the aid of ligroin. Yield: 7 grams. After two recrystallizations from benzene, the substance forms rosettes of needles melting at 103° (corrected).

0.2000 gm. of substance (Kjeldahl) required 9.64 cc. $\frac{N}{10}$ HCl.
Calculated for $C_9H_{10}O_2NCl$: N = 7.02 per cent.
Found: N = 6.76 per cent.

o-Chloroacetylaminobenzyl alcohol and hexamethylenetetramine. The boiling chloroform solution of the components soon deposited an oil which gradually crystallized and set to a solid cake. This was disintegrated and boiled with a little more dry chloroform for about half an hour. As the product thus obtained contained chloroform of crystallization, it was boiled for one hour with dry acetone. The salt melts with decomposition at 144–5°. It is somewhat hygroscopic and is very soluble in water.

0.2245 gm. of substance required 6.9 cc. $AgNO_3$ Solution I.
Calculated for $C_{15}H_{22}O_2N_5Cl$: Cl = 10.44 per cent.
Found: Cl = 10.81 per cent.

o-Chloroacetylaminobenzyl benzoate. The ester was prepared in the usual way from 5 grams of *o*-chloroacetylaminobenzyl alcohol in pyridine and 4 grams of benzoyl chloride. The crude product was washed with dilute sodium carbonate solution and recrystallized from absolute alcohol. Yield: 3.9 grams. Recrystallized first from absolute alcohol and then toluene, the ester forms snow-white needles melting at 113–3.5° (corrected), with slight preliminary softening. It dissolves readily in ether and benzene.

0.1792 gm. of substance required 11.00 cc. $AgNO_3$ Solution II.[7]
Calculated for $C_{16}H_{14}O_3NCl$: Cl = 11.68 per cent.
Found: Cl = 11.42 per cent.

o-Chloroacetylaminobenzyl benzoate and hexamethylenetetramine. The salt crystallized out on adding dry acetone to the chloroform solution and scratching. It darkens above 140° and melts at 154–5° to a thick, greenish liquid.

0.1989 gm. of substance required 8.35 cc. $AgNO_3$ Solution II.
Calculated for $C_{22}H_{26}O_3N_5Cl$: Cl = 7.99 per cent.
Found: Cl = 7.81 per cent.

p-Chloroacetylaminobenzoic acid ethyl ester. This was prepared in the usual way from the amino ester in toluene, chloroacetyl chloride, and normal potassium hydroxide solution. The substance conformed to the description given by Einhorn.[31]

p-Chloroacetylaminobenzoic acid ethyl ester and hexamethylenetetramine. The salt retained chloroform, which was only very slowly given off *in vacuo* at the temperature of boiling alcohol. It is very soluble in water and melts at 155–6° with decomposition.

0.2264 gm. of substance required 5.68 cc. $AgNO_3$ Solution I.
Calculated for $C_{17}H_{24}O_3N_5Cl$: Cl = 9.29 per cent.
Found: Cl = 8.83 per cent.

p-Chloroacetylaminobenzoic acid diethylaminoethyl ester (chloroacetylnovocain). 28 grams of novocain (hydrochloride) were treated with 210 cc. of normal sodium hydroxide solution and

[31] D. R. P. 106502; *Friedlaenders Fortschr. d. Teerfarbenfabrikation,* v, p. 820, 1897–1900.

50 cc. of benzene added to redissolve the free base. To the mixture were added, drop by drop and with cooling and shaking, 20 grams of chloroacetyl chloride. The mixture was made distinctly alkaline with sodium hydroxide and shaken out with ether. The upper layer was dried and concentrated until the ether had distilled off. On adding ligroin cautiously to the benzene solution, this set to a thick mass of microscopic needles. These were filtered off and washed with ligroin. Yield: 21 grams. For analysis, the substance was recrystallized from ligroin and then melted at 63–4.5° (corrected). It does not keep well in the crude state.

0.2078 gm. of substance required 6.7 cc. $AgNO_3$ Solution I.[7]
Calculated for $C_{15}H_{21}O_3N_2Cl$: Cl = 11.34 per cent.
Found: Cl = 11.35 per cent.

p-Chloroacetylaminobenzoic acid diethylaminoethyl ester and hexamethylenetetramine. To the clear chloroform solution were added several volumes of dry acetone. On cooling, the solution set to a thick mass. This was filtered off, washed with acetone, and recrystallized by dissolving in a little chloroform and precipitating with dry acetone. It was not found possible to further purify the salt. It forms lenticular micro-platelets which sinter at 126° and melt at 128–9°.

0.0768 gm. of substance (Kjeldahl) required 9.9 cc. $\frac{N}{10}$ HCl.
0.1744 gm. of substance required 8.76 cc. $AgNO_3$ Solution II.
Calculated for $C_{21}H_{33}O_3N_6Cl$: N = 18.55 per cent; Cl = 7.84 per cent.
Found: N = 18.05 per cent; Cl = 9.34 per cent.

m-Chloroacetylaminoacetophenone. 21.5 grams of *m*-aminoacetophenone were chloroacetylated in the usual manner, the mixture being allowed to become warm in order to ensure complete solution of the amine. After cooling, the chloroacetyl derivative was filtered off and washed with water and toluene. After bone-blacking in benzene, the product was obtained by addition of ligroin to the hot, filtered solution. Yield: 23.5 grams. Recrystallized from absolute alcohol, the substance forms prisms melting at 113.5–14.5° (corrected). It is rather difficultly soluble in cold absolute alcohol, benzene, and ether, more readily in chloroform and acetone. It turns a pale greenish yellow in sulphuric acid and dissolves with a very faint color.

0.3154 gm. of substance (Kjeldahl) required 14.90 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{10}H_{10}O_2NCl$: N = 6.62 per cent.
Found: N = 6.61 per cent.

m-Chloroacetylaminoacetophenone and hexamethylenetetramine. The salt forms micro-platelets which melt with decomposition at 165–6°. It is rather difficultly soluble in water. With absolute alcohol, it changes to a mass of hair-like needles. Sulphuric acid turns the salt yellow and dissolves it with a pale greenish yellow color.

0.2183 gm. of substance required 6.12 cc. $AgNO_3$ Solution I.
Calculated for $C_{16}H_{23}O_2N_5Cl$: Cl = 10.08 per cent.
Found: Cl = 9.94 per cent.

p-Chloroacetylaminoleucomalachite green. 5 grams of amino compound were chloroacetylated in benzene solution in the presence of an excess of two-normal sodium hydroxide solution. It was found convenient to carry out the operation in a separatory funnel, owing to the vigorous shaking required for the decomposition of the difficultly soluble addition products first formed upon addition of the chloride to the mixture. The benzene solution was washed with water, dried over sodium sulphate and evaporated to small bulk. A portion of the dark green syrup thus obtained was taken up in alcohol, water added until slightly turbid, and allowed to stand, with occasional rubbing. The pale greenish micro-crystals finally deposited were recrystallized from absolute alcohol with the aid of a freezing mixture. The compound sinters above 80° and gradually softens to a tar when rapidly heated.

0.1592 gm. of substance (Carius) gave 0.0539 gm. AgCl.
Calculated for $C_{25}H_{28}ON_3Cl$: Cl = 8.41 per cent.
Found: Cl = 8.37 per cent.

p-Chloroacetylaminoleucomalachite green and hexamethylenetetramine. The crude, dark green syrup resulting on evaporation of the benzene layer obtained in the chloroacetylation of 5 grams of amino compound was taken up in 10 cc. of dry chloroform. 2 grams of hexamethylenetetramine were added and the mixture was boiled for one hour. Most of the chloroform was boiled off from the clear green solution and dry acetone added. The solu-

tion was poured off and the residue again treated with dry acetone. On standing over night, the salt separated from the acetone solutions as a nearly colorless powder. In subsequent preparations, it was merely necessary to add dry acetone to a concentrated chloroform solution of the salt and seed the solution with a crystal from the first experiment. The salt melts at 189–90° with decomposition, and is insoluble in water, dissolving readily, however, in dilute mineral acids. Yield: 5.6 grams.

0.2250 gm. of substance gave 0.0587 gm. AgCl.
Calculated for $C_{31}H_{40}ON_7Cl$: Cl = 6.31 per cent.
Found: Cl = 6.45 per cent.

o-Chloroacetylamino-p′, p″-tetraethyldiaminotriphenylmethane and hexamethylenetetramine. Starting with *o*-amino-*p′*, *p″*-tetraethyldiaminotriphenylmethane, the chloroacetyl derivative was obtained as a dark green syrup and was added to hexamethylenetetramine in the same way as in the case of the preceding substance. The chloroform solution was evaporated to dryness *in vacuo*, treated with dry acetone, filtered, and again evaporated *in vacuo*. On adding a mixture of ether and water, the colored impurities were taken up by the ether while the salt remained suspended in the aqueous layer in the form of colorless microplatelets. These were filtered off, washed alternately with water and ether, and dried. The product melts at 177–8° with decomposition and dissolves readily in dilute mineral acids.

0.1739 gm. of substance required 2.8 cc. $AgNO_3$ Solution I.
Calculated for $C_{35}H_{48}ON_7Cl$: Cl = 5.74 per cent.
Found: Cl = 5.67 per cent.

p-Chloroacetylamino-p′,p″-tetraethyldiaminotriphenylmethane and hexamethylenetetramine. The *p*-amino compound was chloroacetylated and the crude product added to hexamethylenetetramine in the same way as in the preceding examples. After boiling off the chloroform, several volumes of dry acetone were added, whereupon the salt crystallized out on scratching. The precipitate of micro-plates was filtered off and washed well with dry acetone, practically all the green color being removed in this way. The salt is insoluble in water, but dissolves readily in one

molecular equivalent of dilute mineral acid. It melts at 168–9°. Yield: 5.5 grams, from 5 grams of amino compound.

0.2131 gm. of substance required 3.6 cc. $AgNO_3$ Solution I.
Calculated for $C_{35}H_{48}ON_7Cl$: Cl = 5.74 per cent.
Found: Cl = 5.95 per cent.

6-Chloroacetylaminoquinoline. 12 grams of 6-aminoquinoline were chloroacetylated in the usual way in benzene solution with chloroacetyl chloride and an excess of normal sodium hydroxide solution. The substance separated as an oil, which soon solidified. This was filtered off, washed with water and benzene, and recrystallized, with bone-blacking, from a mixture of benzene and toluene. Yield: 9.9 grams of minute prisms which melt to a red tar at 153-5° after turning red. The substance is very soluble in alcohol and is very irritating to the mucous membrane.

0.2022 gm. of substance gave 22.42 cc. N (767.5 mm. and 21.5°).
Calculated for $C_{11}H_9ON_2Cl$: N = 12.79 per cent.
Found: N = 12.69 per cent.

The *hydrochloride* was precipitated from the mother liquors from the recrystallization by passing in a stream of hydrochloric acid gas. It was recrystallized by dissolving in 85 per cent alcohol and adding acetone to the warm solution. The salt forms thin, pale red-brown platelets which turn brown above 220°, then darken and sinter, and finally decompose above 250°. It is very irritating to the mucous membrane and is easily soluble in water, yielding an orange solution.

0.1797 gm. of substance required 7.2 cc. $\frac{N}{10}$ NaOH for neutralization.
Calculated for $C_{11}H_9ON_2Cl \cdot HCl$: HCl = 14.19 per cent.
Found: HCl = 14.49 per cent.

6-Chloroacetylaminoquinoline and hexamethylenetetramine. The crude salt was washed well and ground up with dry chloroform and then treated in the same manner with dry acetone. It forms pale drab-colored micro-platelets which dissolve readily in water and melt with decomposition at 203–4°.

0.4316 gm. of substance required 11.63 cc. $AgNO_3$ Solution I.
Calculated for $C_{17}H_{21}ON_6Cl$: Cl = 9.83 per cent.
Found: Cl = 9.49 per cent.

THE QUATERNARY SALTS OF HEXAMETHYLENE-TETRAMINE.

IV. MONOHALOGENACYLATED SIMPLE AMINES, UREAS, AND URETHANES, AND THE HEXAMETHYLENETETRAMINIUM SALTS DERIVED THEREFROM.

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With but one exception, the hexamethylenetetraminium salts described in this paper are purely of aliphatic origin and were obtained from the halogenacyl compounds and hexamethylenetetramine by the methods described in the previous papers. The salts are all easily soluble in water. On standing, more rapidly on warming, the solutions decompose with the liberation of formaldehyde and other products, which, contrary to the behavior observed with the aromatic compounds, remain in solution. A search of the literature has revealed to us the description of the chloroacetyl derivative of but one simple aliphatic amine; *viz.*, chloroacetylallylamine.[1] Our work has necessitated the preparation of the chloroacetyl compounds of several of the simpler amines and the preparation and properties of these will be found below.

EXPERIMENTAL.

(1) *Monohalogenacyl derivatives of simple amines.*

Chloroacetamide may be easily prepared in excellent yield as follows: Ethyl chloroacetate is well cooled in a freezing mixture and vigorously turbined. Two volumes of well cooled, concentrated aqueous ammonia are then added. The reaction occurs smoothly with the formation of a thick paste of the amide. After

[1] C. Harries and I. Petersen: *Ber. d. deutsch. chem. Gesellsch.*, xliii, pp. 635 and 1758, 1910.

standing several hours at room temperature, the product is filtered off, washed, and dried, and then agrees in properties with those described in the literature.

Chloroacetamide and hexamethylenetetramine. This substance was first prepared by Einhorn and Göttler,[2] with whose description our preparation agreed.

α-Iodopropionamide. 27 cc. of concentrated aqueous ammonia were diluted to about 50 cc., placed in a freezing mixture, vigorously turbined, and treated with 30 grams of α-iodopropionyl chloride,[3] added drop by drop. After about half an hour's stirring, the amide was filtered off. In the crude state it turned slightly yellow, but lost its sensitiveness to light after recrystallization from water. Yield: 20.5 grams. Recrystallized from toluene, the amide forms long, glistening needles which soften and turn yellow above 150° and melt at 159-60.5° (corrected). It is difficultly soluble in benzene, toluene, and cold water. The aqueous solution, boiled with a little silver nitrate, gives a copious precipitate of silver iodide.

0.4165 gm. of substance (Kjeldahl) required 21.0 cc. $\frac{N}{10}$ HCl.
Calculated for C_3H_6ONI: N = 7.01 per cent.
Found: N = 7.06 per cent.

Under the conditions used, the amide did not yield a quaternary salt with hexamethylenetetramine.

β–Iodopropionamide. This was prepared from 30 grams of β-iodopropionyl chloride[4] in the same way as the α-compound. In the crude state the amide rapidly turns pink, but loses its sensitiveness to light after bone-blacking and recrystallizing from water. Yield: 20 grams. According to Henry,[5] who prepared the amide from methyl β-iodopropionate and aqueous ammonia, it melts at 100–1°. Our preparation was recrystallized from toluene, forming feathery plates which melted to a yellow liquid at 141.5–2.5° (corrected) with preliminary softening.

[2] A. Einhorn and M. Göttler: *Ann. d. Chem.*, ccclxi, p. 150, 1908.

[3] E. Abderhalden and M. Guggenheim: *Ber. d. deutsch. chem. Gesellsch.*, xli, p. 2855, 1908.

[4] E. Abderhalden and E. Gressel: *Ztschr. f. physiol. Chem.*, lxxiv, p. 476, 1911, and Paper III of this series (this *Journal*, xxi, p. 103, 1915).

[5] L. Henry: *Compt. rend. Acad. d. sc.*, c, p. 117, 1885.

It is difficultly soluble in benzene and toluene and is fairly soluble in cold water.

0.3611 gm. of substance (Kjeldahl) required 18.50 cc. $\frac{N}{10}$ HCl.
Calculated for C_3H_6ONI: N = 7.01 per cent.
Found: N = 7.17 per cent.

β-Iodopropionamide and hexamethylenetetramine. 12 grams of amide and 8.5 grams of base are dissolved in about 750 cc. of commercial acetone, which is then boiled gently for about three hours in a water bath heated to 60–70°. After a short time, the salt begins to separate in the form of broad, glistening needles, and care must be taken to keep within the limits of temperature given in order to avoid superheating on the bottom and sides of the flask and consequent decomposition of the heavy precipitate. Yield: 16.1 grams. The salt melts with decomposition at 169–70°.

0.1769 gm. of substance required 5.28 cc. $AgNO_3$ Solution I.[6]
Calculated for $C_9H_{18}ON_5I$: I = 37.43 per cent.
Found: I = 37.58 per cent.

Chloroacetmethylamide. 13 grams of methylamine hydrochloride (in aqueous solution) were well cooled and treated with 190 cc. of 10 per cent sodium hydroxide solution. The solution was then placed in a freezing mixture, well turbined, and treated, drop by drop, with 23 grams of chloroacetyl chloride. After making slightly acid with hydrochloric acid, the mixture was extracted twelve times with chloroform. This was dried over calcium chloride, evaporated to small bulk, diluted with ligroin, and placed in a freezing mixture. The amide crystallized out and was filtered off in the cold. Yield: 9.3 grams. Recrystallized from dry, alcohol-free ether with the aid of a freezing mixture, the compound forms delicate needles melting at 45–6° (corrected) with preliminary softening. It is easily soluble at room temperature in water and the other usual solvents. When exposed to the air, it slowly volatilizes.

0.2040 gm. of substance (Kjeldahl) required 18.90 cc. $\frac{N}{10}$ HCl.
Calculated for C_3H_6ONCl: N = 13.03 per cent.
Found: N = 12.97 per cent.

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[6] 1 cc. = 0.00352 gm. Cl; 0.01259 gm. I.

Chloroacetmethylamide and hexamethylenetetramine. The salt gradually precipitates on boiling a solution of the components in dry chloroform. When heated, it turns brown and melts with decomposition at 173–4°. It is very soluble in water.

0.2141 gm. of substance required 8.60 cc. $AgNO_3$ Solution I.
Calculated for $C_9H_{18}ON_5Cl$: Cl = 14.32 per cent.
Found: Cl = 14.14 per cent.

Chloroacetdimethylamide. 30 grams of 33 per cent aqueous dimethylamine solution were diluted with an equal volume of water, cooled in a freezing mixture, and treated with 230 cc. of double normal sodium hydroxide solution. 19.6 cc. of chloroacetyl chloride (1.1 mols.) were then added, drop by drop, with vigorous shaking. At the end, the solution was made slightly acid with acetic acid and shaken out six times with ether. This was dried over calcium chloride, evaporated to small bulk, and the residue fractionated *in vacuo.* Yield: 6.6 grams, boiling at 11 mm. at 98.5–9.5° (corrected, thermometer in vapor, bath at 130–5°). The amide crystallizes in a freezing mixture and melts at 15.5° (corrected). It is miscible in all proportions with water and alcohol and is very irritating to the skin.

0.2437 gm. of substance[7] required 37.36 cc. $AgNO_3$ Solution II.[8]
0.5079 gm. (Kjeldahl) required 41.20 cc. $\frac{N}{10}$ HCl.
Calculated for C_4H_8ONCl: N = 11.53 per cent; Cl = 29.17 per cent.
Found: N = 11.37 per cent; Cl = 28.52 per cent.

Chloroacetdimethylamide and hexamethylenetetramine. The clear solution obtained by dissolving the components in dry chloroform and boiling for one hour was evaporated to small bulk. The addition of several volumes of dry acetone resulted in the precipitation of a gum which crystallized on rubbing. The salt was pulverized, boiled with dry acetone, and dried. It melts at 160–2° to an orange liquid and is extremely soluble in water. It also dissolves in chloroform and alcohol.

0.2148 gm. of substance required 15.30 cc. $AgNO_3$ Solution II.
Calculated for $C_{10}H_{20}ON_5Cl$: Cl = 13.55 per cent.
Found: Cl = 13.25 per cent.

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[7] Hydrolysis with alcoholic sodium hydroxide solution.
[8] 1 cc. = 0.00186 gm. Cl.

Chloroacetethylamide. This was prepared in the same way as the dimethyl isomer. 100 grams of 33 per cent aqueous ethylamine solution yielded 71 grams of amide, boiling at 97.5–8.5° at 13 mm. and melting at 22–2.5° (corrected).

0.0830 gm. of substance (Kjeldahl) required 6.9 cc. $\frac{N}{10}$ HCl.
Calculated for C_4H_8ONCl: N = 11.53 per cent.
Found: N = 11.64 per cent.

Chloroacetethylamide and hexamethylenetetramine. The salt separates slowly from a boiling solution of the components in dry chloroform as thick, hexagonal plates. It darkens above 160° and melts with decomposition at 167–8°. It is very soluble in water.

0.2037 gm. of substance required 7.75 cc. $AgNO_3$ Solution I.
Calculated for $C_{10}H_{20}ON_5Cl$: Cl = 13.55 per cent.
Found: Cl = 13.39 per cent.

Chloroacetdiethylamide. The only variation from the procedure employed in the previous cases was that the reaction mixture was extracted ten times with chloroform. 15.2 grams of diethylamine yielded 11 grams of highly refracting liquid boiling at 125.5–30.5° at 21–2 mm., of which most boiled at 126.5–8.5° at 21 mm. It is easily soluble in water but not miscible with it in all proportions. It has a piquant odor which is pleasant at first but soon irritates the mucous membrane.

0.1382 gm. of substance (Kjeldahl) required 9.5 cc. $\frac{N}{10}$ HCl.
Calculated for $C_6H_{12}ONCl$: N = 9.37 per cent.
Found: N = 9.82 per cent.

Chloroacetdiethylamide and hexamethylenetetramine. The solution of the components in dry chloroform was boiled one hour, filtered from a small amount of what was probably hexamethylenetetraminium chloride, and concentrated to small volume. About four volumes of dry acetone were then added, causing the gradual deposition of the salt in glistening rhombs. These were pulverized and boiled with dry acetone. The salt melts at 174° to a turbid liquid which, on further heating, clears up, darkens, and finally effervesces above 190°. It is very soluble in water and also dissolves in chloroform and alcohol.

0.2020 gm. of substance required 7.07 cc. $AgNO_3$ Solution I.
Calculated for $C_{12}H_{24}ON_5Cl$: Cl = 12.27 per cent.
Found: Cl = 12.32 per cent.

Chloroacetpiperidide. 25 grams of piperidine were dissolved in about 150 cc. of dry, alcohol-free ether and cooled with ice water. To the solution was added, drop by drop, with continued cooling and shaking, a solution of 12 cc. of chloroacetyl chloride in dry, alcohol-free ether. After standing a short time, the mixture was shaken successively with small amounts of water, dilute hydrochloric acid, and dilute sodium carbonate solution. It is probable that the chief loss occurred at this point. The ethereal layer was dried over sodium sulphate, evaporated to small bulk, and fractionated *in vacuo*. After two distillations, the substance was obtained as a moderately viscous, practically colorless oil boiling at 12.5 mm. at 143.5–5.5° (corrected, thermometer in vapor, bath at 155–75°), of which the greater part boiled at 144°. Yield: 8.7 grams. It is irritating to the mucous membrane and is soluble in cold water.

0.1924 gm. of substance gave 16.25 cc. N (753 mm. and 26.7°).
Calculated for $C_7H_{12}ONCl$: N = 8.67 per cent.
Found: N = 9.15 per cent.

Chloroacetpiperidide and hexamethylenetetramine. The viscous solution obtained by boiling the components for two hours in a little dry chloroform was filtered, after which it set to a crystalline mass. On adding dry acetone, almost all the salt went into solution, precipitating out again almost immediately. The product was filtered off and boiled for one hour with dry acetone to remove chloroform of crystallization. The salt forms microplatelets which dissolve easily in water. It becomes pasty at 168–70° and melts completely at 184° with gas evolution.

0.1951 gm. of substance required 11.88 cc. $AgNO_3$ Solution II.
Calculated for $C_{13}H_{24}ON_5Cl$: Cl = 11.75 per cent.
Found: Cl = 11.33 per cent.

Methylenebisiodoacetamide. This was prepared from methylenebischloroacetamide[9] and sodium iodide in dry acetone. On

[9] A. Einhorn and T. Mauermayer: *Ann. d. Chem.*, cccxliii, p. 284, 1905.

warming the mixture for a short time sodium chloride precipitated and the new compound separated out. Water was added and the precipitate filtered off and recrystallized twice from acetic acid. It forms arborescent masses of thin plates which darken above 220° when rapidly heated, and decompose at 227–9° with evolution of iodine.

0.2215 gm. of substance (Kjeldahl) required 12.05 cc. $\frac{N}{10}$ HCl.
Calculated for $C_5H_8O_2N_2I_2$: N = 7.34 per cent.
Found: N = 7.62 per cent.

The hexamethylenetetramine addition product of the compound could not be obtained in a state of purity.

Ethylenebischloroacetamide. 15 grams of ethylenediamine hydrochloride were dissolved in water and chloroacetylated in the usual manner with chloroacetyl chloride and sodium hydroxide. The product separated immediately in a state of purity. Yield: 14 grams. Recrystallized first from 85 per cent alcohol, then acetic acid, the substance forms prismatic needles melting at 174–6° (corrected) with preliminary softening. It is difficultly soluble in the cold in the usual solvents.

0.1219 gm. of substance gave 14.15 cc. N (768 mm. and 22.4°).
Calculated for $C_6H_{10}O_2N_2Cl_2$: N = 13.15 per cent.
Found: N = 13.19 per cent.

(2) Chloroacetylureas and urethanes.

Chloroacetylurea and hexamethylenetetramine. Equimolecular amounts of the components were boiled in acetone for four hours. The salt gradually separated in the form of plates which melted with decomposition at 176–7° when freshly prepared, but showed a melting point of 191–2° after standing for some weeks. It is very easily soluble in water.

0.1992 gm. of substance required 7.20 cc. $AgNO_3$ Solution I.
Calculated for $C_9H_{17}O_2N_6Cl$: Cl = 12.82 per cent.
Found: Cl = 12.72 per cent.

Chloroacetylmethylurea[10] *and hexamethylenetetramine.* Equimolecular amounts of the components were dissolved separately in

[10] G. Frerichs: *Chem. Zentralbl.*, lxx, pt. ii, p. 285, 1899.

hot acetone and the solutions mixed. The salt soon began to separate in long needles which were filtered off after forty-eight hours. It sinters at 149–51° and gradually melts, decomposing at 178°. It is very soluble in water.

0.2129 gm. of substance required 7.1 cc. $AgNO_3$ Solution I.
Calculated for $C_{10}H_{19}O_2N_6Cl$: Cl = 12.20 per cent.
Found: Cl = 11.74 per cent.

Chloroacetylbenzylurea. One mol. of benzylurea was suspended in dry benzene and 1.1 mols. of chloroacetyl chloride were added. The mixture was boiled until the evolution of hydrochloric acid ceased, after which the chloroacetyl derivative crystallized out on cooling. Recrystallized from absolute alcohol, it forms glistening needles which melt at 153–5° (corrected). It is difficultly soluble in the cold in absolute alcohol, benzene, chloroform, and acetone.

0.2571 gm. of substance (Kjeldahl) required 22.65 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{10}H_{11}O_2N_2Cl$: N = 12.37 per cent.
Found: N = 12.34 per cent.

Chloroacetylbenzylurea and hexamethylenetetramine. After a few minutes' boiling, the salt separated from the solution of the components in dry chloroform as a mass of long, silky needles. Heating was continued for about fifteen minutes, after which the product was filtered off, washed with chloroform, and boiled with acetone. It is readily soluble in water and melts at 160°.

0.2125 gm. of substance required 5.75 cc. $AgNO_3$ Solution I.
Calculated for $C_{16}H_{23}O_2N_6Cl$: Cl = 9.67 per cent.
Found: Cl = 9.53 per cent.

Chloroacetylurethane[10] *and hexamethylenetetramine.* A solution of the components in dry chloroform was boiled for one-half hour and allowed to stand over night. The salt thus obtained in almost quantitative yield melts at 166–7° and is very soluble in water.

0.2355 gm. of substance required 7.75 cc. $AgNO_3$ Solution I.
Calculated for $C_{11}H_{20}O_3N_5Cl$: Cl = 11.60 per cent.
Found: Cl = 11.58 per cent.

FURTHER EXPERIMENTS ON THE RELATIVE EFFECT OF WEAK AND STRONG BASES ON THE RATE OF OXIDATIONS IN THE EGG OF THE SEA URCHIN.

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When Warburg discovered that NaOH raises the rate of the oxidations in the fertilized eggs of the sea urchin, although it does not enter the egg,[1] he made an important addition to our knowledge of the localization of oxidations in the cell. He correctly concluded that this leaves no doubt that the oxidations raised by NaOH must be located at the surface of the egg. If we are prepared to admit this, we are compelled to face the possibility that the interior of the cell receives little or no oxygen, since whatever oxygen may diffuse to the egg will be chiefly, if not all, used up at the surface of the cells.

In Warburg's observations the weak base NH_4OH had no influence on the rate of the oxidations in the fertilized egg, although it enters the latter. This would be intelligible on the assumption that no oxidations or practically none occur inside the cell, and that the oxidations at the surface of the cell are accelerated by the hydroxyl ion and not by the molecule of the base. There are, however, facts which are not in harmony with both of these conclusions. Unfertilized eggs of the sea urchin as well as of annelids can be induced to develop under the influence of bases, and the bases are only active in the presence of oxygen. The activating action of the bases is also inhibited by the addition of KCN or NaCN.[2] The activation of the egg consists in a change of the surface layer of the egg (which may or may not result in a membrane formation) and the bases bring about this change by accelerating or starting certain oxidations

[1] O. Warburg: *Ztschr. f. physiol. Chem.*, lxvi, p. 305, 1910.

[2] J. Loeb: *Artificial Parthenogenesis and Fertilization*, Chicago, 1913, p. 147 and ff.

in the surface layer or at the surface of the eggs. The writer showed that for this change the weaker bases NH_4OH and the amines are equally, if not more efficient than the strong bases NaOH or tetraethylammoniumhydroxide. We must, therefore, conclude either that in this case the bases accelerate oxidations not through the OH ion but by the influence of the molecule, or that, if the effect of bases on oxidations is due to the OH ion, oxidations cannot be confined to the surface of the egg but must take place also inside the egg or at least in the cortical layers adjacent to the surface of the egg.

It was found also that the maximum effect of the weaker bases was reached at a comparatively low concentration, while that of the strong bases increases steadily with the concentration.[3] This could speak in favor of either possibility. We felt that further experiments would be required to clear up this ambiguity. We chose for our purpose the fertilized egg instead of the unfertilized, since in the latter the objection may be raised that the increase of the rate of oxidations by bases is merely an indirect one and due to the activation of the egg by the base. We selected the egg of *Arbacia*.

Method and results.

Eggs of *Arbacia* were fertilized in sea water, and then washed three times or more in a neutral mixture of $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$) in the proportion in which these salts occur in the sea water. Next a homogeneous suspension of them was made. This suspension was divided into several equal parts. One part remained in the neutral $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$) solution; to the other were added varying amounts of NaOH or NH_4OH, and the rate of oxidations for one hour was determined by Winkler's method. The percentage increase of the rate of oxidations of the eggs in the alkali over those in the neutral solution was thus ascertained.

The following two tables give the results of the experiments, and the results are expressed graphically in the two curves of Fig. 1. In this figure the abscissae give the amount of cc. of $\frac{N}{10}$ base added to 100 cc. of $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$), and the ordinates represent the rise in the rate of oxidations above that at

[3] J. Loeb and H. Wasteneys: this *Journal*, xiv, p. 355, 1913.

the neutral point. Two facts are salient from these data; namely, first, that the effect of NH_4OH is considerably greater than we should expect if both NaOH and NH_4OH acted alike (*e.g.*, only at the surface of the egg) and if both acted merely through their OH ion concentration; and second, that the curves of the effects of both run closely parallel. The falling off of the rate of oxidations if the concentration of the base exceeds a certain limit may be accounted for by a secondary effect and may remain outside of the discussion.

The fact that NaOH acts more powerfully than NH_4OH excludes the idea that we are dealing here with an effect of the

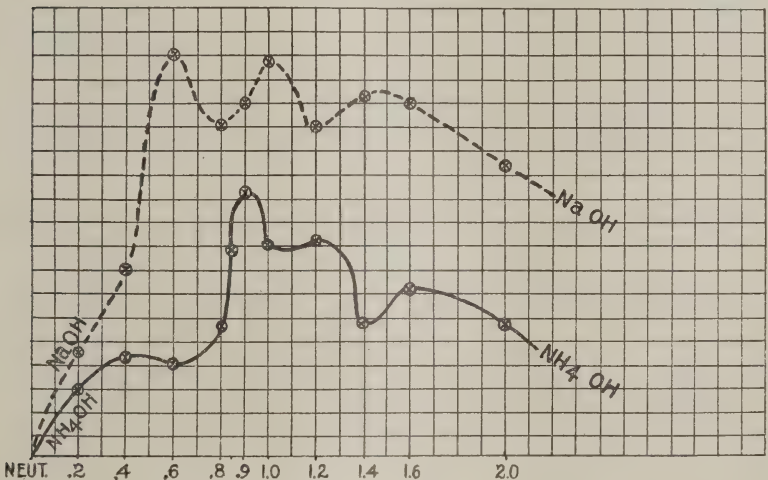

FIG. 1.

undissociated molecule; and the parallelism in the effects of the two bases suggests a common source of their action. Such a common source could exist only in the OH ion (if the action of the molecule is excluded). The fact that the weak base acts more powerfully than we should expect from its degree of dissociation indicates that this effect is overcompensated by an advantage which the weak base has. This may be the fact that the weak base diffuses into the egg while the strong base does

not.[4] In this case one can explain all the phenomena on the assumption that while the greater part of oxygen is consumed at the surface of the egg, some diffuses into the egg and is consumed in the layers adjacent to the surface; and the more so the nearer the surface these layers are. While the weak base can accelerate the rate of the oxidations both at the surface and beneath the

TABLE I.

Increase in the rate of oxidations of the fertilized egg of Arbacia through the influence of NaOH. Temperature 20° C.

NO. OF EXPERIMENT	NATURE OF SOLUTION	OXYGEN CONSUMED	COEFFICIENT OF OXIDATIONS
		mg.	
I	Neutral $\frac{M}{2}$ $(NaCl + KCl + CaCl_2)$..................	0.75	1.00
	100 cc. $\frac{M}{2}$ $(NaCl + KCl + CaCl_2)$ + 1.0 cc. $\frac{N}{10}$ NaOH	2.01	2.68
	100 cc. $\frac{M}{2}$ $(NaCl + KCl + CaCl_2)$ + 1.6 cc. $\frac{N}{10}$ NaOH	1.87	2.49
II	Neutral $\frac{M}{2}$ $(NaCl + KCl + CaCl_2)$..................	0.75	1.00
	100 cc. $\frac{M}{2}$ $(NaCl + KCl + CaCl_2)$ + 0.2 cc. $\frac{N}{10}$ NaOH	1.08	1.44
	100 cc. $\frac{M}{2}$ $(NaCl + KCl + CaCl_2)$ + 0.8 cc. $\frac{N}{10}$ NaOH	1.82	2.42
	100 cc. $\frac{M}{2}$ $(NaCl + KCl + CaCl_2)$ + 1.4 cc. $\frac{N}{10}$ NaOH	1.90	2.53
III	Neutral $\frac{M}{2}$ $(NaCl + KCl + CaCl_2)$..................	0.51	1.00
	100 cc. $\frac{M}{2}$ $(NaCl + KCl + CaCl_2)$ + 2.0 cc. $\frac{N}{10}$ NaOH	1.14	2.24
	100 cc. $\frac{M}{2}$ $(NaCl + KCl + CaCl_2)$ + 0.9 cc. $\frac{N}{10}$ NaOH	1.28	2.50
	100 cc. $\frac{M}{2}$ $(NaCl + KCl + CaCl_2)$ + 0.6 cc. $\frac{N}{10}$ NaOH	1.29	2.53
	100 cc. $\frac{M}{2}$ $(NaCl + KCl + CaCl_2)$ + 1.2 cc. $\frac{N}{10}$ NaOH	1.15	2.25
IV	Neutral $\frac{M}{2}$ $(NaCl + KCl + CaCl_2)$..................	0.47	1.00
	100 cc. $\frac{M}{2}$ $(NaCl + KCl + CaCl_2)$ + 0.4 cc. $\frac{N}{10}$ NaOH	0.85	1.80

surface layer the strong bases are confined in their action to the surface. This would account for the relatively excessive efficiency of the weak base.

[4] We have thought also of the possibility of a specific effect of the weak base, that would presuppose the action of the undissociated molecule (which we have discarded) or of the kation; but since we know of the effect of the OH ion, the assumption of an effect of the kation to the exclusion of the OH ion seemed rather forced.

We must then still explain the discrepancy between Warburg's observation and ours. Warburg found no effect of NH_4OH on the rate of oxidation of the fertilized egg, but this can be accounted for by the fact that he worked on the egg of a different species. Our results in the egg of *purpuratus* differ also from those in *Arbacia*. In the fertilized egg of *purpuratus* the NaOH acted

TABLE II.

Increase in the rate of oxidations of the fertilized egg of Arbacia through the influence of NH_4OH. Temperature 20° C.

NO. OF EXPERIMENT	NATURE OF SOLUTION	OXYGEN CONSUMED	COEFFICIENT OF OXIDATIONS
		mg.	
I	Neutral $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$)	0.51	1.00
	100 cc. $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$) + 0.6 cc. $\frac{N}{10}$ NH_4OH	0.72	1.41
	100 cc. $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$) + 1.2 cc. $\frac{N}{10}$ NH_4OH	0.98	1.92
	100 cc. $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$) + 1.4 cc. $\frac{N}{10}$ NH_4OH	0.81	1.59
II	Neutral $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$)	0.64	1.00
	100 cc. $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$) + 0.8 cc. $\frac{N}{10}$ NH_4OH	1.01	1.58
	100 cc. $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$) + 1.0 cc. $\frac{N}{10}$ NH_4OH	1.22	1.91
III	Neutral $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$)	0.32	1.00
	100 cc. $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$) + 1.6 cc. $\frac{N}{10}$ NH_4OH	0.55	1.72
IV	Neutral $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$)	0.68	1.00
	100 cc. $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$) + 0.4 cc. $\frac{N}{10}$ NH_4OH	0.98	1.44
	100 cc. $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$) + 0.9 cc. $\frac{N}{10}$ NH_4OH	1.44	2.12
V	Neutral $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$)	0.73	1.00
	100 cc. $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$) + 0.2 cc. $\frac{N}{10}$ NH_4OH	0.95	1.30
	100 cc. $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$) + 0.85 cc. $\frac{N}{10}$ NH_4OH	1.38	1.89
VI	Neutral $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$)	0.51	1.00
	100 cc. $\frac{M}{2}$ ($NaCl + KCl + CaCl_2$) + 2.0 cc. $\frac{N}{10}$ NH_4OH	0.80	1.57

only when it was used in a very high concentration ($>10^{-3}$ N), in which it must have had an etching effect on the surface;[5] while in the egg of *Arbacia* it acted in a much lower concentration.

[5] Loeb and Wasteneys: *loc. cit.*, p. 459.

We are not in a position to account for these specific differences in different eggs.

If our conclusion is correct it compels us to consider not only the surface but also the layers adjacent to the surface of a cell as the seat of oxidations. According to this view the greater rate of oxidations at the surface would be due to the fact that the greater part of the available oxygen is used up here so that comparatively little can diffuse into the cell.

THE DEVELOPMENT OF ALKALINITY IN GLOMERELLA CULTURES.

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(From the Laboratories of Plant Pathology and Bacteriology, Virginia Agricultural Experiment Station, Blacksburg. Paper No. 31.)

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In an article recently published[1] the writer showed that the fungus, *Glomerella rufomaculans*, gives rise in the course of its growth to alkaline conditions in the medium after the process of autolysis sets in. It was shown that the conditions of alkalinity arose whether the fungus grew upon its natural host, the apple fruit, or upon artificial media in the laboratory.

A study of some species of Gloeosporium by Krüger[2] reports the same results when the fungus was grown upon solutions containing peptone, either with or without dextrose, especially where the fungus made considerable growth. The production of alkaline conditions in culture media has been for a long time known in the study of bacteriology.

The present paper gives the results of experiments designed to study further this question. The fungus used was *Glomerella rufomaculans*, the cause of the bitter rot of apples. The method of making the cultures and the nutrient solution used have been described in a former article.[3]

The original Czapek's culture solution is slightly acid in reaction, but becomes alkaline as the result of the activities of *Glomerella*. A titration of the original medium showed that 0.2 cc. of $\frac{N}{10}$ alkali was necessary to neutralize 100 cc. of solution. Titration of the medium after a culture had grown upon it for a month showed that 12.0 cc. of $\frac{N}{10}$ acid was necessary for neutralization. Another culture developed alkalinity equal to 18.4

[1] H. S. Reed: *Virginia Agricultural Experiment Station, Annual Report* 1911–12.

[2] F. Krüger: *Arb. a. d. biol. Anst. Land-. u. Forstwirtsch.*, ix, p. 233, 1913.

[3] Reed: this *Journal*, xix, p. 257, 1914.

cc. $\frac{N}{10}$ alkali in forty-five days. Two cultures which had grown three months and had developed a very dark color in the medium were titrated after sufficient dilution to permit the use of an indicator. One culture showed an alkalinity of 14.4 cc. $\frac{N}{10}$ alkali per 100 cc.; the other, 14.8 cc. $\frac{N}{10}$ alkali.

The first study[4] showed that a still greater degree of alkalinity might arise when the fungus was grown upon media of different composition. The fungus developed an alkalinity per 100 cc. equal to 42.8 cc. of $\frac{N}{10}$ acid on a medium consisting of the following:

	gm.
Ammonium phosphate	3.0
Potassium nitrate	1.0
Magnesium sulphate	0.5
Lactose (or dextrose)	5.0
Distilled water	100.0 cc.

In attempting to discover the causes of this alkalinity, several possible conditions were open for investigation: (1) the formation of carbonates; (2) the formation of free ammonia; and (3) the formation of organic bases.

The formation of carbonates would arise from the action of the fungus upon the sugar present in the medium. Sodium carbonate would be especially likely to be formed through the differential absorption of ions by the fungus. In this case where $NaNO_3$ was supplied the fungus would absorb the nitrate ions more rapidly than the sodium ions. The sodium ion would then unite with any CO_2 present with the formation of a carbonate.[5]

In the first place cultures were analyzed for the sodium and potassium. It was found that nearly all the amounts added were still in the culture medium after the fungus had grown for three months. An uninoculated control which required 1.8 cc. of decinormal alkali for neutralization, *i.e.*, was acid, had 81 mg. of sodium and 70.3 mg. of potassium. The average of two cultures showed an alkalinity equal to 24.1 cc. of decinormal acid and had 70.5 mg. of sodium and 66.7 mg. of

[4] Reed: *Virginia Agricultural Experiment Station, Annual Report*, p. 76, 1911–12.

[5] For studies on this action see Kohn and Czapek: *Beitr. z. chem. Phys. u. Path.*, viii, p. 302, 1906. Reed: *Ann. Botany*, xxi, p. 501, 1907.

potassium. This shows that enough ions were present to form soluble carbonates.

The amount of carbonates present in the culture medium was next ascertained. For this purpose cultures of different ages were filtered through paper and the clear medium was analyzed. A number were analyzed by the Fresenius-Classen method and others in the Parr total carbon apparatus. The results are shown in Table I.

TABLE I.

Carbonates in media on which Glomerella had grown.

AGE	CONDITION	METHOD	CO_2
			gm.
4 mos.	Much darkened	Fresenius-Classen	0.1289
4 "	" "	" "	0.0649
1 "	White	" "	0.1385
1 "	Darkened	" "	0.1330
1 "	"	" "	0.1095
4 "	White	" "	0.1445
1 "	"	Parr	0.0716
1 "	"	"	0.0901
1 "	"	"	0.0822
1 "	Darkened	"	0.093

These results show that considerable carbon dioxide is formed by the action of the fungus or its enzymes, although as previously noted the fermentation is not active enough to cause any measurable loss in weight of the culture.[6]

It was stated above that the average amount of Na found by analysis was 70.5 mg. If this were combined as carbonate it would require 67 mg. CO_2, an amount which is considerably lower than those found by analysis of several of the cultures. However, ammonium and potassium carbonates, as well as sodium carbonate, would undoubtedly be formed.

It was next found that the CO_2 content influences the titrations made to determine acidity, as the following data show. A culture two months old was filtered and washed free of adhering solution. 100 cc. of the medium were then titrated, using methyl red as indicator. 11.7 cc. of decinormal acid were re-

[6] Reed: *Virginia Agricultural Experiment Station, Annual Report*, p. 70, 1911–12.

quired. The solution was boiled for several minutes and again titrated, when it required 1.6 cc. of additional acid to bring it to the neutral point, or 13.3 cc. of decinormal acid in all.

A second sample of 100 cc. was boiled and then titrated. It required 9.7 cc. of decinormal acid. After a second boiling it required 0.9 cc. more acid, or a total of 10.6 cc. This difference may be due to the fact that ammonium carbonate was present in the solution and was lost by boiling.

It was next found that some of the alkalinity arose from the formation of ammonia. It was noted that Krüger found the most alkalinity when peptone (an ammonifiable compound) was present in the culture medium. The culture medium mentioned in a foregoing paragraph on which an alkalinity of 42.8 cc. decinormal was developed contained a rather large amount of ammonium phosphate.

In the experiments where *Glomerella* was grown on Czapek's medium the formation of ammonia would be most likely to arise from the autolysis of fungous protein, since it was shown in a former article[7] that this fungus produces several enzymes capable of acting upon protein.

The attempt was made to study the denitrifying action of *Glomerella* by cultivating it upon Giltay and Aberson's solution. The fungus grew so poorly, however, on this medium that no results were obtained.

The formation of ammonia can be demonstrated by distilling the fungous cultures with magnesium oxide. To study the process of ammonia formation from week to week, a series of cultures was analyzed weekly with the results given in Table II.

These figures show that after the third week there is a considerable production of ammonia in the cultures. Since the cultures themselves had individual variations the figures necessarily vary somewhat. The general trend of the analysis is to be taken, therefore, rather than individual results.

It will be seen that the nitrate is rapidly used up and that there is an increase of nitrogen in the mycelium during the first weeks of growth. Later the nitrogen content of the mycelium falls off and the organic N and ammonia N of the culture medium

[7] Reed: *Ibid.*

increase. This is taken to mean that the ammonia arises from the autolysis of fungous protein and not from denitrification. Ammonia is undoubtedly lost from the solutions either as ammonium carbonate or as free ammonia, consequently the figures do not represent the entire amount of ammonia produced. However, a part of the alkalinity developed is undoubtedly due to this formation of ammonia.

TABLE II.

Distribution of nitrogen in Glomerella cultures.

WEEKS	IN MYCELIUM	IN SOLUTION		
	Total N	Organic N	Ammonia N	Nitrate N
0	——	——	——	40.00
3	29.30	9.90	0.30	0.65
4	31.60	6.70	0.68	0.50
5	19.50	15.60	3.08	Trace
6	18.77	22.10	1.96	"
7	20.60	13.71	3.50	——
8	18.70	15.00	4.70	——
9	18.92	12.88	1.96	——
10	17.94	13.42	2.10	——

The formation of organic bases as a cause of alkalinity in the culture medium has been investigated and the results have already been published.[8] It was found that bases of the hexone and purine series are developed when *Glomerella* is grown on a synthetic medium like that described above, as a result of the autolysis of fungous protein.

The cause of the alkalinity observed may therefore be referred to three factors: (a) the formation of carbonates; (b) the formation of ammonia; and (c) the formation of organic bases.

[8] Reed: this *Journal*, xix, p. 257, 1914.

GASTRO-INTESTINAL STUDIES. VIII.

A METHOD FOR THE QUANTITATIVE ESTIMATION OF TRYPSIN IN THE GASTRIC CONTENTS.

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(Received for publication, March 29, 1915.)

In the course of investigations on the enzymes found in the gastric contents we decided to employ the method for the estimation of trypsin in the gastric contents advocated by Ehrenreich.[1]

This reads as follows: Five reagent tubes containing gastric juice (1.0, 0.5, 0.25, 0.12, and 0.06 cc.). To each tube add a drop of phenolphthalein solution, and, drop by drop, 10 per cent solution of sodium carbonate, or, better, sodium bicarbonate, until a light red color is produced. Then add to each tube 2 cc. of a 0.2 per cent casein solution made as follows: 0.4 gm. casein dissolved in 40 cc. of $\frac{N}{10}$ NaOH, add 130 cc. of distilled water, and then 30 cc. of $\frac{N}{10}$ HCl. This solution will keep for a week under chloroform. Incubate for two and one-half hours. Add drop by drop a solution of

Glacial acetic acid	1 part
Alcohol (95 per cent)	50 parts
Distilled water	50 parts.

The tubes in which digestion has taken place remain clear; in the others turbidity appears. The clear tubes are marked plus; the opalescent one, plus-minus; and the turbid tubes, minus.

Our results obtained by following carefully the technique above described were invariably negative. It was first evident that the 10 per cent sodium bicarbonate solution failed to give accurate neutralization of the acidity of the gastric contents, since the carbon dioxide rapidly evolved from the strong carbonate solution interferes with the end-point of the phenolphthalein color change. The carbonic acid being a stronger acid than

[1] M. Ehrenreich: *Ztschr. f. klin. Med.*, lxxv, p. 233, 1912.

phenolphthalein prevents the production of the pink color until all the carbon dioxide has been removed. Furthermore, the alkaline concentration of the 10 per cent sodium bicarbonate solution is so great that in gastric contents of low acidity one drop often renders the liquid too strongly alkaline. These objections were remedied by using a 2 per cent solution of sodium bicarbonate, by which the neutralization is well controlled. Other indicators were experimented with, but phenolphthalein has proven the most satisfactory, since, on acidifying to precipitate the undigested casein, the liquid becomes colorless, thus not interfering with the determination of turbidity.

Our results still failed to show digestion except in a few instances, presumably because the amount of casein was too great to allow complete digestion in any but exceptional cases. We therefore reduced the quantity of casein solution one-half and obtained better results.

Finally, by extending the incubation period to five hours at 40° C., instead of the two and one-half hour period, uniform results were obtained. Since by the fractional method [2 to 7] of examination of the gastric contents but 5 cc. are removed at each period, we have used 0.5 cc. of gastric contents for the tryptic determination instead of the 1 cc., advised by Ehrenreich, so that sufficient material will remain for further tests.

The method as modified has yielded most satisfactory results and is hereby given in detail.

[2] M. E. Rehfuss: *Am. Jour. Med. Sc.*, p. 848, 1914.

[3] M. E. Rehfuss, O. Bergeim, and P. B. Hawk: Gastro-Intestinal Studies. I. The Question of the Residuum Found in the Empty Stomach, *Jour. Am. Med. Assn.*, lxiii, p. 11, 1914.

[4] Rehfuss, Bergeim, and Hawk: Gastro-Intestinal Studies. II. The Fractional Study of Gastric Digestion with a Description of Normal and Pathologic Curves, *ibid.*, lxiii, p. 909, 1914.

[5] Bergeim, Rehfuss, and Hawk: Gastro-Intestinal Studies. III. Direct Demonstration of the Stimulatory Power of Water in the Human Stomach, this *Journal*, xix, p. 345, 1914.

[6] Rehfuss and Hawk: Gastro-Intestinal Studies. IV. Direct Evidence of the Secretion of a Gastric Juice of Constant Acid Concentration by the Human Stomach, *Jour. Am. Med. Assn.*, lxiii, p. 2088, 1914.

[7] Rehfuss: Gastro-Intestinal Studies. VI. The Impossibility of Interpreting the Findings Obtained by the Customary Examination of the Test Meal, *ibid.*, lxiv, p. 569, 1915.

(a) Five reagent tubes, Nos. 1, 2, 3, 4, and 5; more if desired.

To tubes 1 and 2 add 0.5 cc. of gastric contents (filter if cloudy).

(b) To tubes 2, 3, 4, and 5 add 0.5 cc. of distilled water.

(c) From tube 2 remove 0.5 cc. of its mixed contents and add to tube 3. Mix thoroughly and add 0.5 cc. from tube 3 to tube 4. Repeat for tube 5.

We now have dilutions of gastric contents of 1, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, and $\frac{1}{16}$.

(d) To each tube add one drop of phenolphthalein solution (phenolphthalein 1 gm.; alcohol (95 per cent) 100 cc.); then add drop by drop a 2 per cent sodium bicarbonate solution until a light pink color is produced.

(e) To tubes 1, 2, 3, and 4 add 0.5 cc. of casein solution. Tube 5 must receive 1 cc. of casein solution, since it contains 1 cc. of the diluted gastric contents. For the casein solution, dissolve 0.4 gm. of casein in 40 cc. of $\frac{N}{10}$ NaOH. Add 130 cc. of distilled water, then 30 cc. of $\frac{N}{10}$ HCl. This leaves the solution alkaline to the extent of 10 cc. of $\frac{N}{10}$ NaOH, minus about 3 cc. neutralized by the casein.

(f) Incubate for five hours at 40° C.

(g) Precipitate the undigested casein by dropwise addition of a solution of the following composition: glacial acetic acid 1 cc., alcohol (95 per cent) 50 cc., distilled water 50 cc. The tubes in which digestion has been complete remain clear; others become turbid.

(h) The tryptic values are expressed in terms of dilution. Thus, complete digestion in tube 3 (a dilution of $\frac{1}{4}$) shows four times the tryptic power of undiluted gastric juice; taken as a standard as 1, therefore, its tryptic value is 4.

(i) Controls of boiled gastric contents plus casein solution, and of distilled water plus casein solution, treated as above stated, must show no digestion, and become turbid on addition of the precipitating solution.

THE EFFECT OF ACID ADMINISTRATION ON PARATHYROID TETANY.

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The extirpation of the parathyroid glands from animals causes the development of a condition of tetany with accompanying abnormalities of metabolism about which our knowledge is but fragmentary. Two theories have been proposed to account for the characteristic symptoms. The first assumes that tetany is caused by a toxic substance which is neutralized, destroyed, or prevented from being formed by the normal functioning of the parathyroid glands. The second theory proposes that the tetany is due to a hyperexcitability of the nerves caused by a deficiency of calcium in the blood and tissues.

The latter hypothesis was advanced by MacCallum and Voegtlin,[1] who first showed that the introduction of soluble calcium salts into an animal in tetany relieves the condition. Further investigations by MacCallum and his coworkers[2,3] furnish supporting evidence, but as yet no definite cause for the deficiency of calcium has been determined.

Relief from tetany may be brought about by the introduction of strontium, magnesium, and barium salts, as well as calcium.[4,5] Joseph and Meltzer[6] showed that injections of a hypertonic

[1] W. G. MacCallum and C. Voegtlin: *Jour. Exper. Med.*, xi, p. 118, 1909.

[2] W. G. MacCallum and K. M. Vogel: *ibid.*, xviii, p. 618, 1913.

[3] W. G. MacCallum, R. A. Lambert, and K. M. Vogel: *ibid.*, xx, p. 149, 1914.

[4] W. N. Berkeley and S. P. Beebe: *Jour. Med. Research*, xx, p. 149, 1909.

[5] C. Voegtlin and W. G. MacCallum: *Jour. Pharm. Exper. Therap.*, ii, p. 421, 1910–11.

[6] D. R. Joseph and S. J. Meltzer: *ibid.*, ii, p. 361, 1910–11.

sodium chloride solution relieve the acute symptoms. Carlson and Jacobson[7] found that greatly hypertonic sugar solutions have the same effect and act by decreasing the excitability of the nervous tissues, an effect also ascribed to the action of calcium and strontium salts. An indirect action on the nerve centers may be brought about by vaso-dilatation produced by the injection of tissue extracts, albumoses, amyl nitrite, and by stimulation of depressor nerves. Only a temporary relief, in some cases lasting but a few hours, is brought about by these treatments.

In connection with the toxin theory of the causation of tetany, ammonia has received considerable attention as a possible factor. When injected in suitable quantities, ammonium salts produce a tetany somewhat similar to that observed after parathyroidectomy. This finding has been correlated with data produced by several investigators showing an increased quantity of ammonia in the blood and urine. A high ammonia content of the blood in parathyroidectomized animals has been observed by MacCallum and Voegtlin,[1] Medwedew,[8] and Jacobson,[9] but the later figures of Carlson and Jacobson[7] and those of Greenwald[10] failed to show an increase. A high ammonia excretion in the urine and an increased ratio of ammonia to total nitrogen were reported by MacCallum and Voegtlin, Underhill and Saiki,[11] Cooke,[12] Berkeley and Beebe,[4] and Falta and Kahn.[13] Greenwald found a smaller variation from the normal.

Many suggestions have been advanced to account for the increased ammonia excretion in the urine and the high ammonia content of the blood. There may be an increased protein catabolism with an excessive ammonia production. The urea-forming power of the liver and other tissues may be damaged and cause a piling up of ammonia. A decreased oxidation may cause an acidosis with a characteristic ammonia formation to neutralize the acids formed. The appearance of lactic acid in the urine,

[7] A. J. Carlson and C. Jacobson: *Am. Jour. Physiol.*, xxviii, p. 133, 1911.
[8] A. Medwedew: *Ztschr. f. physiol. Chem.*, lxxii, p. 410, 1911.
[9] C. Jacobson: *Am. Jour. Physiol.*, xxvi, p. 407, 1910.
[10] I. Greenwald: *ibid.*, xxviii, p. 103, 1911.
[11] F. P. Underhill and T. Saiki: this *Journal*, v, p. 225, 1908–09.
[12] J. V. Cooke: *Jour. Exper. Med.*, xiii, p. 439, 1911.
[13] W. Falta and F. Kahn: *Ztschr. f. klin. Med.*, lxxiv, p. 108, 1911.

as shown by Cooke, has been used to support the last suggestion.

The possibility that there may be a condition of or tendency toward an alkalosis after parathyroidectomy has received no attention. This hypothesis is interesting in connection with the following facts. Alkalies are known to increase the symptoms of tetany. Sodium bicarbonate has been shown to be distinctly harmful, and sodium and potassium acetates apparently act in the same way. Sodium chloride is innocuous in isotonic solution. We shall present evidence to show that the introduction of acids relieves the tetany. We may conclude, therefore, that an artificial alkalosis is unusually harmful, while a condition of acidosis produced by acid introduction is beneficial. The latter conclusion is supported by the observation that an acidosis developed by prolonged fasting tends to diminish the severity of the symptoms. We are at present studying the condition of tetany from this point of view and hope to determine whether the above mentioned tentative hypothesis is justified.

We wish to present in this communication, as a preliminary report, the results of our experiments on the introduction of acids into dogs in relieving parathyroid tetany. The action is quite definite as the period of relief is more prolonged than that observed after the introduction of many of the agents previously used. On account of the great variations in animals and the spontaneous periodicity of the symptoms, we give below sufficient protocols to show the efficiency of acids, introduced intravenously or *per os*, in relieving the symptoms of tetany in dogs.

PROTOCOLS.

No. 12. Adult male bull dog. Weight, 10.4 kg.

Jan. 12. Operation; complete thyroparathyroidectomy.

Jan. 16–19. One acute attack observed each day. Definite mild tetany in intermediate periods.

Jan. 20. Fed 500 cc. of milk. Mild tremors.

Jan. 21. Fed 20 gm. of casein in 200 cc. of water, a.m. Violent tetany at 5 p.m. Injected intravenously 90 cc. $\frac{M}{3.7}$ hydrochloric acid in $\frac{M}{10}$ sodium chloride solution. During injection dog became quiet, panting ceased, and muscles became relaxed. After injection completely relaxed, no tremors. Walked a few steps and lay down. Blood showed no hemolysis. 8 p.m. Lay curled up; no tetany apparent; walked easily.

Jan. 22. No signs of tetany all day, walked about easily. Fed 30 gm. of casein in 300 cc. of water.

Jan. 23, 9 a.m. Occasional slight twitching. Appeared bright. Exertion caused period of moderate contractions.

Jan. 24. Fed 20 gm. of casein in 250 cc. of 0.5 per cent HCl. Depression marked; no tremors.

Jan. 25. Fed 20 gm. of casein in 250 cc. of water. Depression marked, wound showed infection; animal sacrificed. Autopsy: negative except for congestion of viscera. (Water was given by sound, 250 cc. daily except when otherwise stated.)

In this experiment the intravenous injection of 90 cc. $\frac{M}{3.7}$ hydrochloric acid in $\frac{M}{10}$ salt solution, or 8.6 cc. per kg., completely relieved for at least twenty-four hours a tetany which had been observed daily for six days previously. Following this period only mild contractions were observed. The animal was unusually strong, and only during the last two days was the depression sufficient to render the observations of doubtful value.

No. 16. Female bull dog. Weight, 7.9 kg.

Jan. 22. Removed anterior two-thirds of each thyroid with parathyroids.

Jan. 24, 9 a.m. Severe tetany. 11.55 a.m. Injected intravenously 54 cc. $\frac{M}{3.7}$ HCl in $\frac{M}{7}$ NaCl solution. Respirations decreased from 230 to 100 per minute; otherwise no definite improvement. 1.40 p.m. Marked improvement. Tremors barely felt. Walked a little stiffly. 5.30 p.m. Appeared normal in every respect. Blood showed slight hemolysis.

Jan. 25. No tetany. Ate 55 gm. of meat. 200 cc. of water *per os.* Vomited some.

Jan. 26. No tetany.

Jan. 27. No tetany until 9 p.m., when mild tremors were observed. Stiff and did not walk well. 200 cc. of water *per os.*

Jan. 28. Mild tremors during day. 200 cc. of water *per os.*

Jan. 29, 9 a.m. Slight tremors. Gave 2 gm. of sodium bicarbonate *per os* in 200 cc. of water. 2.30 p.m. Marked tremors. Teeth chatter. 4 p.m. Tetany marked. Died after injecting intravenously 50 cc. $\frac{M}{3.7}$ HCl in $\frac{M}{7}$ NaCl solution. Autopsy: congestion of viscera, pregnant.

An improvement was noted soon after the intravenous injection of 6.8 cc. per kg. of a $\frac{M}{3.7}$ HCl solution in $\frac{M}{7}$ saline, and no symptoms of tetany were observed for seventy-eight hours subsequently. Only slight muscular tremors were then apparent until the tetany was aggravated by the introduction of sodium bicarbonate *per os.*

No. 18. Young male dog. Weight, 10.2 kg.

Jan. 26. Removed right thyroid and anterior two-thirds of left thyroid with parathyroids.

Jan. 28. Acute tetany. Muscular cramps, stiffness, lost control of legs, but no tremors.

Jan. 29. Improved. Ate 100 gm. of meat.

Jan. 30, a.m. Slight tremors. 10.55 p.m. Acute tetany. Dog restless, groaning. Began intravenous injection of $\frac{M}{3.7}$ HCl in $\frac{M}{7}$ NaCl solution. 11.30 p.m. 45 cc. injected. Dog much improved. Violent symptoms subsided. 12.06 a.m. 86 cc. introduced. Injection stopped. Animal completely relaxed and quiet. Walked easily.

Feb. 1. No tetany. Ate 100 gm. of meat.

Feb. 2, 3, 4. No tetany. Would not eat.

Feb. 4. Found dead at 11.30 p.m. Autopsy: bronchopneumonia.

Tetany was relieved during the injection of 8.4 cc. $\frac{M}{3.7}$ HCl in $\frac{M}{7}$ NaCl solution per kg., and was not observed during the remainder of the experiment.

No. 19. Adult male cur. Weight, 10 kg.

Jan. 27. Complete thyroparathyroidectomy.

Jan. 29. Mild tetany.

Jan. 30, 3 p.m. Violent tetany. 4 p.m. 26 cc. $\frac{M}{3.7}$ HCl in $\frac{M}{7}$ NaCl solution injected intravenously. No improvement, respirations 208. 4.20–5.00 p.m. 37.5 cc. subcutaneously. Tremors the same, respirations 180. 5.35 p.m. Began intravenous injection again. Tremors the same, respirations 30. 6.00 p.m. 16 cc. injected. Dog becoming relaxed. 6.25 p.m. 37 cc. injected. Stopped injection. Slight tremors felt. Bright, walks with ease. 8 p.m. No tremors felt.

Jan. 31. No tremors apparent during day.

Feb. 1. No tremors apparent during day. Walked stiffly, apathetic.

Feb. 2, 10 a.m. Much brighter. Ate 100 gm. of meat eagerly. Necrosis at points of subcutaneous injection. 11.15 a.m. Mild tremors. During afternoon 650 cc. 0.5 per cent HCl were given *per os*, of which 225 cc. were vomited. Mild tremors became less apparent.

Feb. 3, 9 a.m. Tetany severe. 10.30 a.m. 0.5 gm. HCl in 150 cc. of water. 2 p.m. Tremors less marked. 0.5 gm. HCl in 100 cc. of water. 5.00–5.45 p.m. No tremors felt.

Feb. 4. No tremors during day; depression.

Feb. 5. Faint occasional tremors visible. Depression, necrosis marked. Sacrificed.

Tetany was relieved by the intravenous injection of 10 cc. $\frac{M}{3.7}$ HCl solution in saline per kg., and no recurrence of symptoms was observed for sixty-three hours. The results of dilute

acid ingestion by mouth are not conclusive, but indicate a favorable action in relieving tetany.

No. 25. Young female cur. Weight, 6.7 kg.

Feb. 2. Complete thyroparathyroidectomy. 6 p.m. 0.5 gm. HCl in 150 cc. of water *per os*.

Feb. 3, 10 a.m. 0.5 gm. HCl in 200 cc. of water *per os*. Ate 100 gm. of meat eagerly.

Feb. 4, 9.30 a.m. 100 gm. of meat. 12 noon. Violent tetany. Injected intravenously 61 cc. $\frac{M}{3}$ lactic acid in $\frac{M}{13}$ NaCl solution. Dog improved; ran around room, but tremors still marked. 4 p.m. Tetany improved. 5 p.m. Only faint tremors felt.

Feb. 5. Lay curled up most of the time, perfectly relaxed, no tremors felt. When roused, she ran around easily, after which faint tremors were barely apparent. Condition the same until 5 p.m., when found in definite acute tetany. 8 p.m. No tremors. Walked a little stiffly. 11 p.m. Bright, active, ate crust of bread eagerly, no tremors.

Feb. 6, a.m. No tremors. Appeared about normal. 2 p.m. Slight tremors. 4 p.m. Acute tetany. Injected intravenously 50 cc. $\frac{M}{7}$ HCl in $\frac{M}{7}$ NaCl solution. Tetany relieved during injection. Alert, slightly ataxic; faint tremors only apparent in temporal muscles. 6.20 p.m. No tremors. 7.45. No tremors. Jumped easily into cage.

Feb. 7. Respiration slightly labored. No tremors. Lively. 1 p.m. Slight tremors barely felt.

Feb. 8. Very faint occasional tremor felt. Muscles relaxed. Vomited when water was given. Ran around easily.

Feb. 9. Lively. No tremors all day. Vomited when water was given.

Feb. 10. 9.45 a.m. Mild tremors in head.

Feb. 11. Depressed. No tremors. 2 p.m. Found dead. Autopsy: very small patch of bronchopneumonia.

It is uncertain whether the improvement after the lactic acid injection can be ascribed to the acid introduced owing to the short period of relief and the extreme periodicity of the symptoms. The effects of the hydrochloric acid injection were much more definite and prolonged. With the exception of short periods of mild tremors on the second and fourth days, there was no sign of tetany during the remainder of the experiment.

No. 31. Female bull dog. Weight, 7.8 kg.

Jan. 15, a.m. Removed right thyroid and anterior portion of left with parathyroids.

Jan. 16, a.m. Slight occasional tremors, salivation marked.

Jan. 17, 9 a.m. Depressed. Mild tremors. 1.30 p.m. Extreme tetany. Panting, restless. 1.53 p.m. Started intravenous injection of $\frac{M}{7}$ HCl

in water. Respirations 280. 2.33 p.m. 20 cc. injected. Much quieter. 2.50 p.m. Respirations 200, shallow. Muscles relaxed, no tremors. 3.11 p.m. 38 cc. introduced; injection stopped. Respirations normal. Occasional faint tremors in shoulder muscles. Lay with eyes closed. 3.40 p.m. Placed on floor; looked sleepy, but took an interest in surroundings. Occasional tremor barely apparent. 4.00 p.m. Walked around normally, drank a little water. No tremors. 5.30 p.m. No tremors. Blood showed slight hemolysis. 11.30 p.m. No tremors.

Jan. 18, 9 a.m. Lively. Faint tremors. 11.20 a.m. Acute tetany. 11.35 a.m. Prostrated. Periods of marked contractions. Mild tremors throughout remainder of day.

Jan. 19. Marked tremors. 1.10 p.m. Acute tetany. Found dead at 3 p.m.

The injection of 4.8 cc. per kg., of a $\frac{M}{7}$ hydrochloric acid solution produced a definite though temporary relief from tetany, but did not ward off a severe attack on the following day. We have observed other cases where relief was obtained by the injection of a small quantity of $\frac{M}{7}$ hydrochloric acid, but the tetany again became evident on the following day.

No. 27. Male cur. Weight, 10 kg. Acid introduced in every case per os with sound.

Feb. 5. Complete thyroparathyroidectomy.

Feb. 7, 5 p.m. Mild tremors. 200 cc. 0.25 per cent HCl solution.

Feb. 8, 9.30 a.m. Period of marked tremors in fore legs and head. 10 a.m. Mild occasional tremors. 200 cc. 0.25 per cent HCl solution. 2.30 p.m. Occasional tremor when excited, none when lying in cage. 200 cc. 0.25 per cent HCl solution. 5 p.m. No tremors. 200 cc. 0.25 per cent HCl solution vomited.

Feb. 9, 9.30 a.m. 200 cc. 0.25 per cent HCl solution. No tremors noticed during day.

Feb. 10, 9.30 a.m. 200 cc. 0.25 per cent HCl solution. No tremors. 2.45 p.m. Faint tremors in head felt once. Ran easily up stairs. 200 cc. 0.25 per cent HCl solution. When interested acted as a normal dog, otherwise dull. 5 p.m. 115 cc. of vomitus found.

Feb. 11, 9 a.m. Found lying quietly. On arising, faint contractions in head and neck for half a minute. 200 cc. 0.25 per cent HCl solution. Ran around easily. 2 p.m. Faint tremors barely apparent. Otherwise completely relaxed. 4 p.m. 200 cc. 0.25 per cent HCl solution.

Feb. 12, 9.30 a.m. 200 cc. 0.25 per cent HCl solution. Occasional faint tremors felt during day. 5.30 p.m. 200 cc. 0.25 per cent HCl solution.

Feb. 13, a.m. No tremors. 200 cc. of *water*. 3 p.m. Mild tremors, apparently more marked. Head shook violently at times. 200 cc. of water.

Feb. 14. Depressed. Tremors mild but more marked than previously. 10.15 a.m. Gave 2 gm. $NaHCO_3$ in 200 cc. of water *per os*. 10.45 a.m. Restless. Tremor attacks more violent. 11 a.m. Two attacks of vigorous contractions in fifteen minutes. 11.40 a.m. Violent contractions; vomited small amount of fluid. Continuous mild tremors with periods of marked contractions. Tetany observed seemed considerable in view of the weak condition of the dog.

Feb. 15. 4 gm. $NaHCO_3$ in 200 cc. of water. Vomited about half. Mild tremors. Depression.

Feb. 16. Dog moribund. Sacrificed.

Unlike most dogs, the animal showed little tendency to vomit, and for this reason became a valuable subject for treatment with acid by mouth. As soon as the tetany was apparent, acid was given *per os* in an attempt to relieve the condition. By the ingestion of 400 cc. of a 0.25 per cent hydrochloric acid solution (or less when vomited) per day, the symptoms of tetany were all but completely effaced. Only occasional faint tremors were observed during the five days of acid ingestion. After this period water was given instead of acid and the tetany became more apparent at once. On account of the state of depression into which the animal had fallen, no violent tetany was observed, so that sodium bicarbonate was given on the following day. This caused a more acute condition than had been observed at any time previously. The experiment contrasts very sharply the beneficial effects of acid introduction and the harmful action of the ingestion of bases. The relief produced by the introduction of acid by mouth is apparently much less permanent than that brought about by intravenous injections.

No. 22. Young male bull dog. Weight, 7.4 kg.

Jan. 30. Right thyroid and anterior two-thirds of left removed.

Feb. 1. Ate 200 gm. of meat. 11 p.m. Mild tetany. Dilute hydrochloric acid by mouth vomited with a little meat. Repeated with 100 cc. 0.5 per cent HCl.

Feb. 2, 10 a.m. 65 cc. of vomitus in bottle. No tetany; refused meat. 100 cc. 0.5 per cent HCl solution *per os*. 12 noon. Much livelier. Appeared normal. 2 p.m. Ate half a slice of bread. 8 p.m. Ate half a slice of bread. No tremors observed during day.

Feb. 3, 9 a.m. No tremors. 2 p.m. Occasional tremor felt in head. Depressed. 5 p.m. 0.5 gm. HCl in 150 cc. of water *per os*. Slight tremors. 11 p.m. No tremors.

Feb. 4. Mild tetany throughout day. Vomited practically all the acid given.

Feb. 5. Mild tremors. Retained 100 cc. 0.25 per cent HCl solution. 5 p.m. Injected intravenously 100 cc. $\frac{M}{7}$ NaCl solution. No improvement.

Feb. 6, 2.30 p.m. Severe tetany. Animal died during acid injection. Autopsy: bronchopneumonia throughout lungs.

Dilute hydrochloric acid by mouth definitely relieved tetany for one and one-half days. Thereafter sufficient acid was not retained to prevent the appearance of tremors, but acute attacks were never observed. The intravenous injection of $\frac{M}{7}$ NaCl solution caused no improvement.

As may be seen from the above protocols, the intravenous injections of $\frac{M}{3.7}$ or $\frac{M}{7}$ hydrochloric acid solutions relieved parathyroid tetany in dogs. The period elapsing before the return of the symptoms varied somewhat according to the amount of acid introduced, but in some cases extended over several days. We have purposely used acids of considerable strength in order to keep the volume of liquid injected at a minimum. The injection of acid in salt solution apparently decreases the tendency toward harmful effects, as we have had more evident reactions by introducing hydrochloric acid in pure water than in salt solution. That the injection of 100 cc. of a $\frac{M}{7}$ sodium chloride solution alone has no apparent beneficial effect has been observed by others as well as ourselves.

In support of the evidence offered by acid injection, we have observed relief in several cases when acid was introduced *per os*. The difficulties of this procedure, however, render it of uncertain value. The stomachs of most dogs in tetany are so sensitive that no material can be retained. We were fortunate to obtain one animal which, by careful manipulation, retained most of the acid introduced, and during the period of acid ingestion (five days) showed no typical tetany. The symptoms became acute later when sodium bicarbonate was given.

The relief of parathyroid tetany by the administration of acids suggests the possibility of a beneficial action due to a variation in the acid-base equilibria in the body, and offers a new point of view for the study of this and allied conditions.

THE INFLUENCE OF CERTAIN VEGETABLE FATS ON GROWTH.[1]

By E. V. McCOLLUM and MARGUERITE DAVIS.

(*From the Laboratory of Agricultural Chemistry of the University of Wisconsin, Madison.*)

(Received for publication, March 27, 1915.)

In previous papers[2] we have pointed out that when rats have been brought through a period of successful growth on diets of purified food constituents, but containing no fats, failure sooner or later supervenes. If, however, such diets of suitable make-up contain certain fats, there is no failure of nutrition, and reproduction and rearing of the young have been observed. Up to the present time we have obtained positive results with butter fat, egg yolk fats, kidney fat (ether-soluble portion of kidney free from visible fat), and the ether extract of the ripe testicle of the codfish. To this list Osborne and Mendel have added commercial cod liver oil,[3] and more recently[4] the more liquid portion of beef fat. In our experience commercial lard, olive oil, tallow, and cottonseed oil have given negative results. Osborne and Mendel found negative results likewise with cold pressed almond oil.[5] Up to the present, therefore, the existence in the vegetable world of the substances which are carried by certain animal fats has not been demonstrated. Experiments with carefully prepared plant fats are still few in number and it is evident that no conclusions can be drawn from negative results obtained by feeding commercial fats or oils about whose treatment we know nothing, since heating to fairly high temperatures in pressing and subsequent bleaching are by no means uncommon.

[1] Published with the permission of the Director of the Wisconsin Experiment Station.

[2] E. V. McCollum and M. Davis: this *Journal*, xv, p. 167, 1913; xix, p. 245, 1914; xx, p. 641, 1915. Compare T. B. Osborne and L. B. Mendel: *ibid.*, xvi, p. 423, 1913–14.

[3] Osborne and Mendel: *ibid.*, xvii, p. 401, 1914.

[4] Osborne and Mendel: *Proc. Soc. Exper. Biol. and Med.*, xii, p. 92, 1915.

[5] Osborne and Mendel: this *Journal*, xvii, p. 401, 1914.

Since it is now clear that with the fat-free ration which we have used with such excellent results[6] the only factor which is wanting can be supplied by the fats from certain sources, and by no other proximate constituent of foodstuffs, it follows that any foodstuff of plant origin which can supplement this ration and lead to normal growth without subsequent failure, or lead to recovery after the animals have begun to fail, must carry the constituents which are associated with the animal fats which produce the same effects. We have adopted this method of comparing the biological properties of the fats carried by corn (maize), wheat, wheat embryo, rye, rolled oats, and dried pig heart and kidney. The results leave no room for doubt that certain fats occurring in the plant kingdom possess just as pronounced stimulating action on growth as do certain animal fats, although this is by no means true of plant fats from all sources.

Experimental methods.

The rats whose curves are shown in Charts I to IX were fed the fat-free diet of casein, milk sugar, dextrin, agar-agar, and salts until in most instances marked loss of weight had occurred and the animals were in an enfeebled condition. This required usually between twenty and twenty-five weeks. In fact, a considerable number of animals died at the time the additions were being made to the diet. When in a state of enfeeblement and emaciation on the fat-free diet, the ration was changed to fat-free ration 50 per cent, and plant product 50 per cent. The results were surprising: the rats which received the corn meal addition responded at once with increase in weight and marked change in appearance (Chart I). Inflamed eyes which were evident in many of the animals soon became normal in appearance.[7] The coats became glossy and the animals assumed a normal, well fed appearance. The recovery is still apparently complete nine weeks after the change was made in the food. Like corn, wheat embryo, when added to the fat-free ration, is capable of rescuing rats from the threshold of death when brought to the point of failure as the result of feeding the diet deprived of those constituents carried by certain fats (Chart II).

[6] McCollum and Davis: *ibid.*, xx, p. 641, 1915.

[7] Osborne and Mendel have pointed out that a similar eye condition is relieved by feeding butter fat (*ibid.*, xvi, p. 431, 1913).

In marked contrast to these vegetable foods, the entire wheat kernel when added to the extent of 50 per cent of the diet, serves only to save the animals from immediate death, but does not enable them to increase in body weight and assume a normal appearance (Chart III). Even after six weeks with the wheat addition, without appreciable increase in weight, a substitution of wheat embryo for the wheat led at once to pronounced increase in body weight and improvement in appearance.

When rye replaced one-half of the customary fat-free diet of rats which were at the point of failure, we have seen considerable gain in weight in one individual, and a slight temporary gain in two others. There was no marked improvement in the appearance of these rats as the result of feeding rye (Chart IV).

Similarly we have not been able to restore rats in the condition described by adding rolled oats to the extent of one-half of the diet, although with this addition, as with wheat and rye, some of the animals are saved from immediate death (Chart V). Our negative results with these grains must not be accepted as final, however, since the rats were in very feeble condition and may have been too far depleted to admit of recovery. In one instance, however, there was a distinct improvement following a change from oats to corn (Chart V).

Other points on which we are prepared to furnish evidence are:

1. That 50 per cent of corn added to the fat-free diet is vastly superior to 5 per cent butter fat when the animals have been brought to a point near which failure of nutrition would set in (Chart VI).

2. That the addition of 5 per cent wheat or 5 per cent corn meal is not sufficient to prevent decline and death on the fat-free diet (Chart VII).

We include also for comparison with these vegetable additions the curves of rats which under similar circumstances were changed from the fat-free diet to a mixture of (a) dried pig heart, dextrin, and calcium lactate (Chart VIII), and (b) dried pig kidney, dextrin, and calcium lactate (Chart IX). We were greatly surprised to observe the very temporary benefit derived from feeding the pig heart as compared with the kidney.

Both the hearts and kidneys were obtained fresh from animals slaughtered on the Experiment Station farm, and were passed through a hasher and dried in the same set of trays at tempera-

tures not above 70° C. in a current of air. There was no bacterial decomposition of either preparation. We would not draw the conclusion from these results that the essential lipins or their accompanying unknown substances are absent from heart muscle. It is possible that some other factor is involved here which contributed to the failure of these rats. Nevertheless, the prompt response with rapid growth, when dried kidney replaced the heart tissue affords a striking example of the paucity of our knowledge regarding the peculiar physiological properties of our commonest foodstuffs.

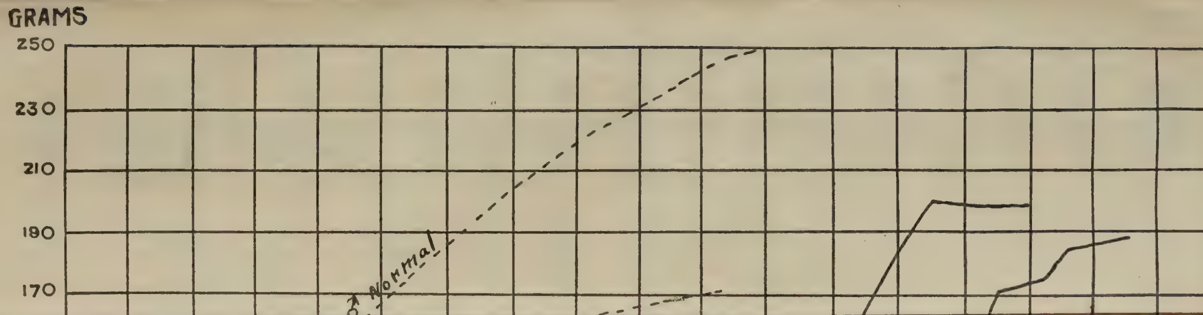
We have not been successful in obtaining reproduction in rats which have been so badly depleted by prolonged feeding of a fat-free ration, after recovery resulting from fat additions. We must, therefore, reserve conclusions as to the efficiency of the plant fats on which we report in this paper for inducing reproduction.[8] Young rats must be given these rations with the vegetable additions from the beginning in order to obtain evidence on this point.

The effects of wheat, rye, and oats seem to suggest that the differences observed in their effects on depleted animals may well be due to quantitative differences in the yield of the unknown accessory substances under consideration, rather than to an entire absence of the same. This viewpoint is strongly supported in the case of the wheat kernel as compared with wheat embryo. Observations with swine[9] indicate that a ration composed wholly of the corn kernel (70 per cent corn meal and 30 per cent gluten feed) and a suitable salt mixture, will lead to growth and reproduction, while a ration made of other grains, containing but 30 per cent of corn meal, and a suitable salt mixture fails to induce growth. Possibly this finds a partial explanation in the quantitative differences in the rations of unknown accessory substances needed for growth.

The possibility should also be emphasized that there may be carried by certain of the grains substances sufficiently toxic to cause injury to animals in such an enfeebled condition.

[8] At the time of reading proof on this article we have secured two litters of young from the rats receiving the corn addition. One of these litters is alive at the age of five days.

[9] E. B. Hart and E. V. McCollum: *ibid.*, xix, p. 373, 1914.



sponsible for the beneficial effect of this food, for it has not been found possible to produce this recovery with any of the proximate constituents of foodstuffs other than fats from certain sources. All these rats appear to be fairly well nourished after nine weeks with the corn meal addition.

The composition of the fat-free ration was as follows:

	per cent
Casein	18.0
Dextrin	56.3
Lactose	20.0
Agar-agar	2.0
Salt mixture	3.7

COMPOSITION OF THE SALT MIXTURE

	gm.
NaCl	0.173
$MgSO_4$(anhydrous)	0.266
$NaH_2(PO_4) + H_2O$	0.347
K_2HPO_4	0.954
$CaH_4(PO_4)_2H_2O$	0.540
Calcium lactate	
$Ca (C_3H_5O_3)_2 + 5 H_2O$	1.300
Fe lactate (Merck)	0.118

The normal expectation of growth is represented in these charts by a broken line.

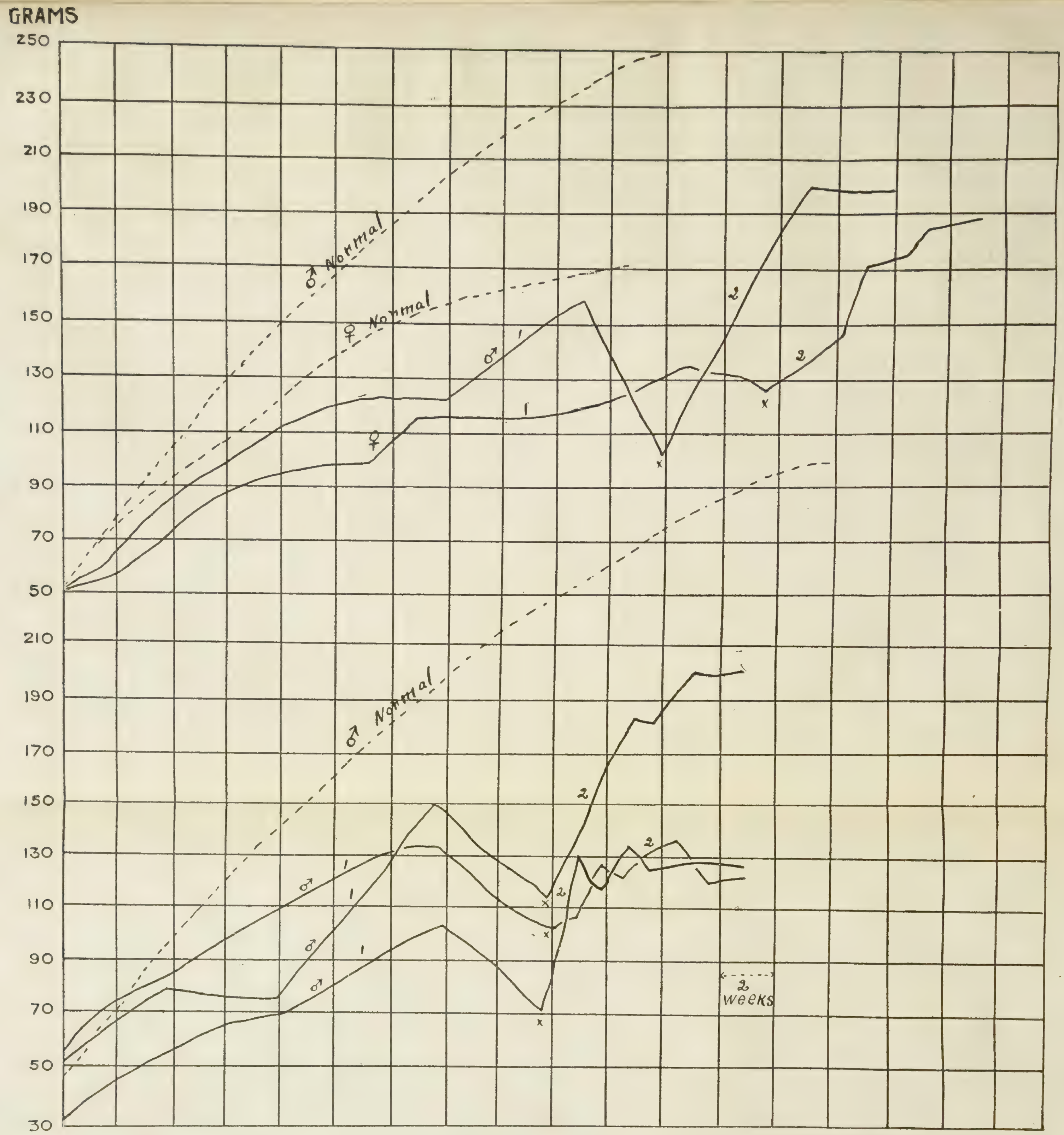


CHART I. Illustrates the effect of feeding corn meal to rats which had been brought to a state of great emaciation and feebleness by prolonged feeding of a fat-free diet.

Period 1. Fat-free diet.
Period 2. Fat-free diet 50 per cent, corn meal 50 per cent.

These animals would have died within a few days if kept on the fat-free diet. The prompt recovery when corn meal is fed in addition to the fat-free diet, is in every respect similar to the effects observed when butter fat, egg yolk fat, kidney fat, cod liver oil, etc., is introduced into the ration. The fats carried by the corn meal are evidently responsible for the beneficial effect of this food, for it has not been found possible to produce this recovery with any of the proximate constituents of foodstuffs other than fats from certain sources. All these rats appear to be fairly well nourished after nine weeks with the corn meal addition.

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Calcium lactate	
Ca $(C_3H_5O_3)_2 + 5\ H_2O$	1.300
Fe lactate (Merck)	0.118

The normal expectation of growth is represented in these charts by a broken line.

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Period 1

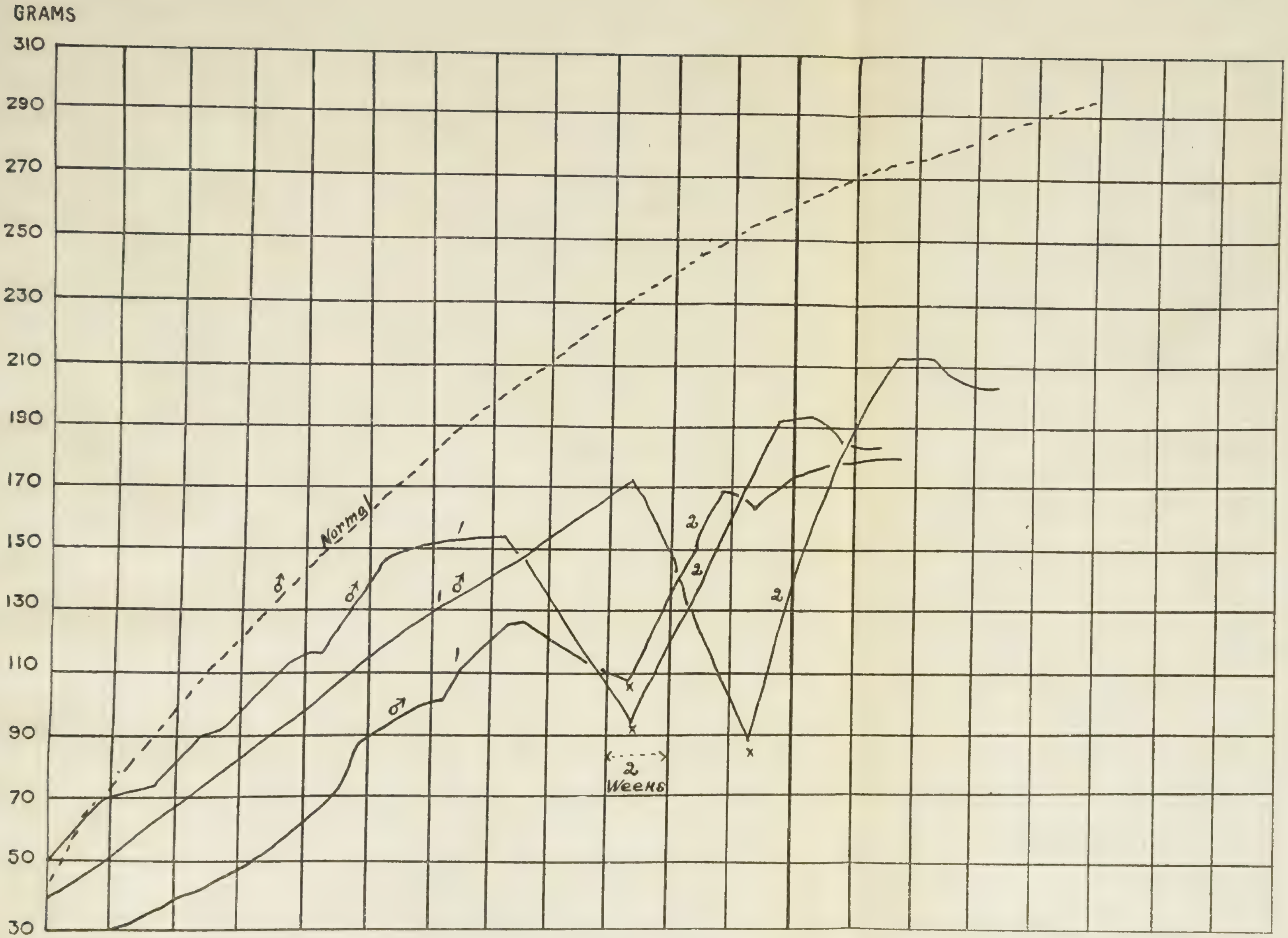
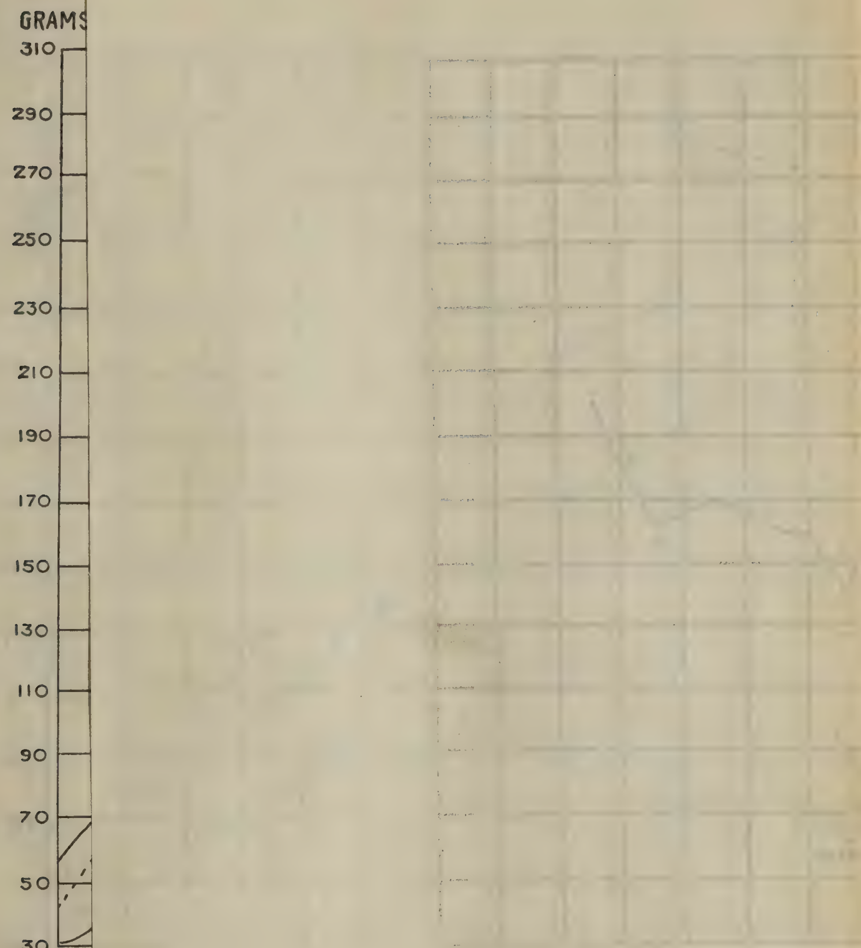


CHART II. Shows the prompt recovery of rats as the result of the introduction of wheat embryo into the diet, after they were fed a fat-free diet until in a moribund condition. It is evident that the specific effects hitherto observed as the result of feeding butter fat, egg fats, etc., are obtainable by feeding plant products from certain sources.

Period 1. Fat-free diet. Period 2. Fat-free diet 50 per cent, wheat embryo 50 per cent.



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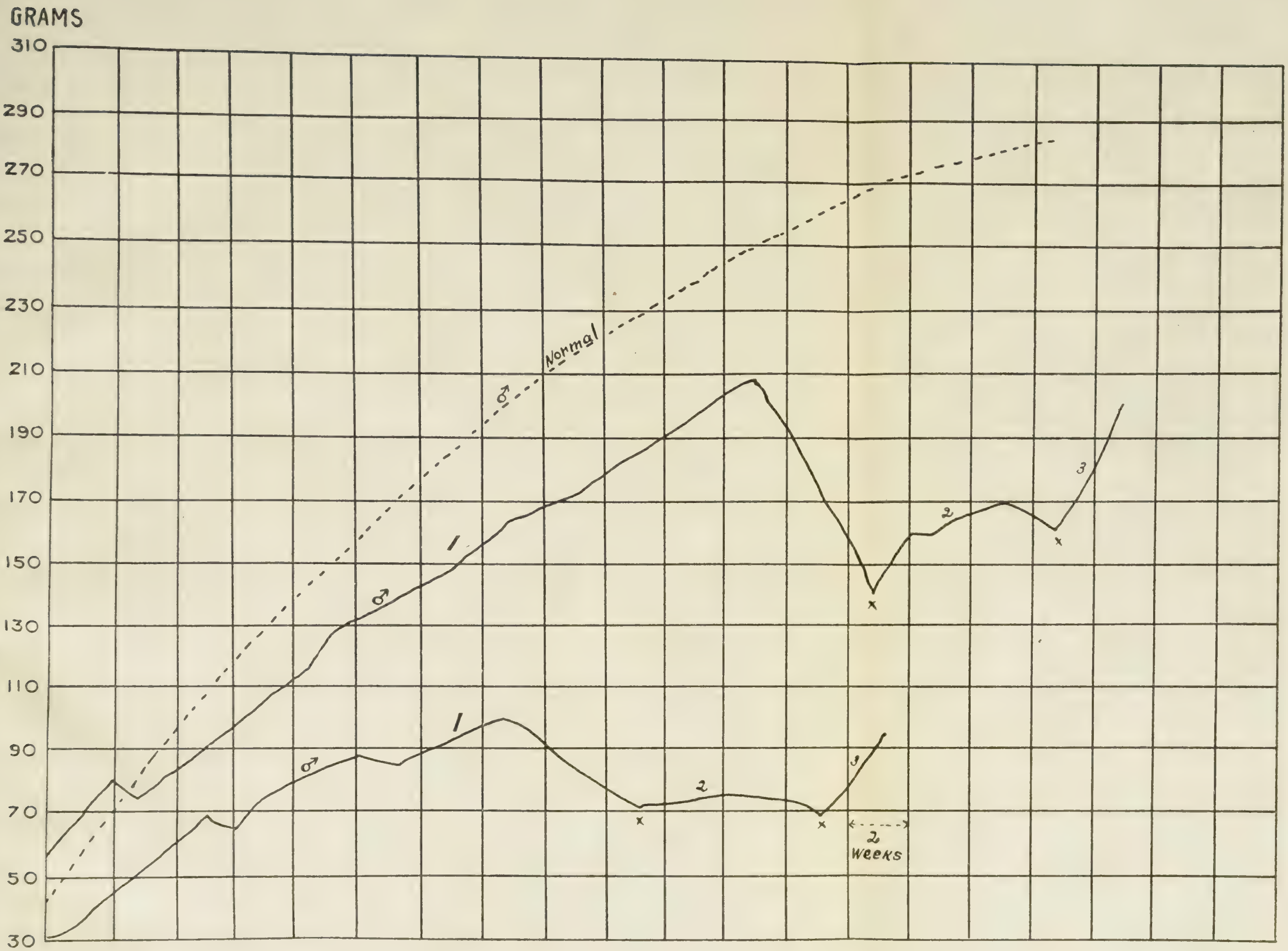

CHART III. Shows how the introduction of 50 per cent whole wheat meal into the fat-free diet employed in this series saves the animals from immediate death, but does not permit the prompt increase in weight which results from adding corn meal in the same proportion instead of wheat. In Period 3 wheat embryo was substituted for the whole wheat, when growth was at once resumed.

Period 1. Fat-free diet.
Period 2. Fat-free diet 50 per cent, wheat 50 per cent.
Period 3. Fat-free diet 50 per cent, wheat embryo 50 per cent.

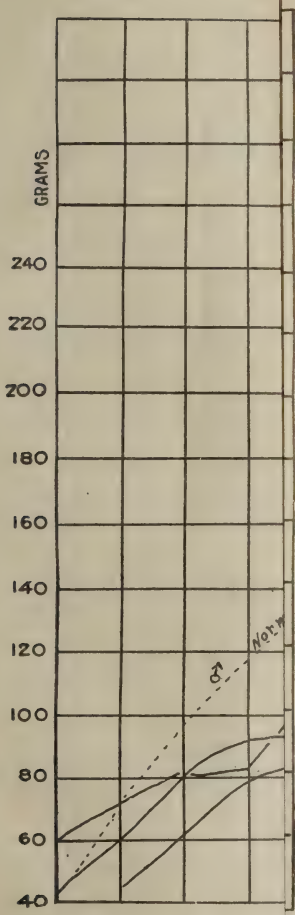

Chart IV. Shows the h
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Period 1. Fat-free ration.

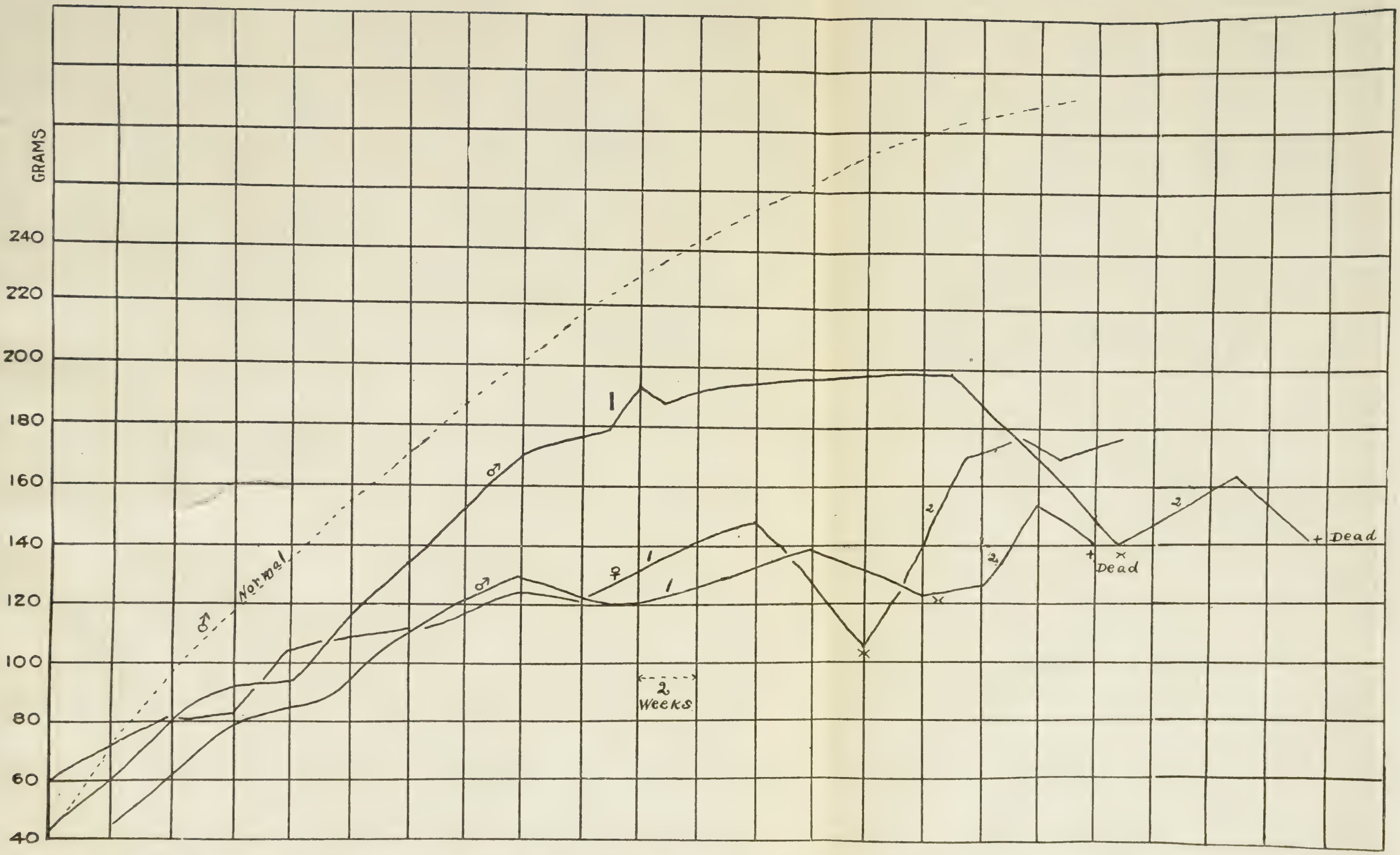

CHART IV. Shows the effects of feeding rye after rats had begun to fail on the fat-free diet. Despite the fact that considerable growth resulted in one case, this rat did not show as marked improvement in appearance as did the rats receiving corn meal (Chart I). Rye serves to prevent immediate death. It would appear that rye fats carry the unknown accessory substances present in butter fat, and some other fats, but in too small amount for complete recovery.

Period 1. **Fat-free ration.** Period 2. Fat-free ration 50 per cent, rye 50 per cent.

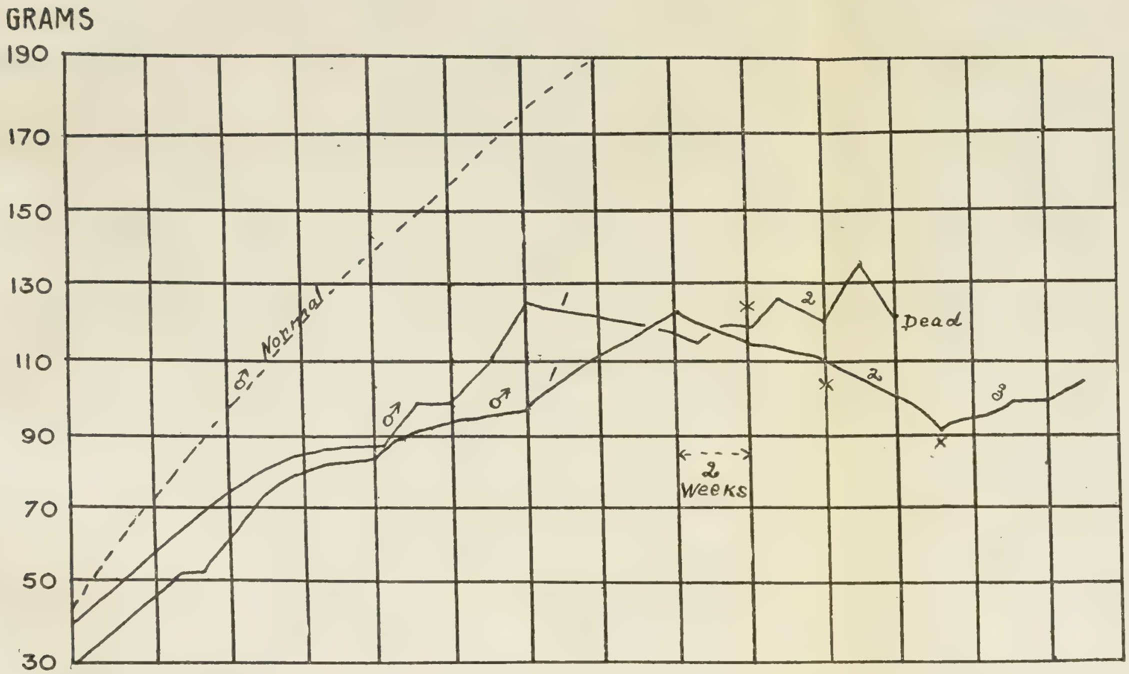

CHART V. Illustrates the effects of introducing rolled oats into the fat-free diet after failure had set in on the latter food. But little if any improvement has been observed as a result of feeding oats to such animals. We should accept these negative results with reserve, since the rats were in a very feeble state, but the noticeable improvement in one instance which followed the change from oats to corn meal points to the inferiority of the fats of the oat kernel as compared with corn in promoting growth.

Period 1. Fat-free ration.
Period 2. Fat-free ration 50 per cent, rolled oats 50 per cent.
Period 3. Fat-free ration 50 per cent, corn 50 per cent.

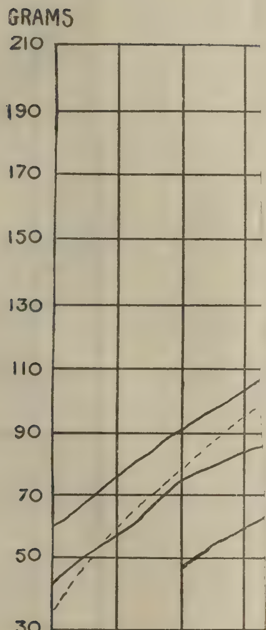

CHART VI. Furnishe
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Period 1. Fat-free ration.
Period 2. Fat-free ration + 5
Period 3. Fat-free ration 50 p

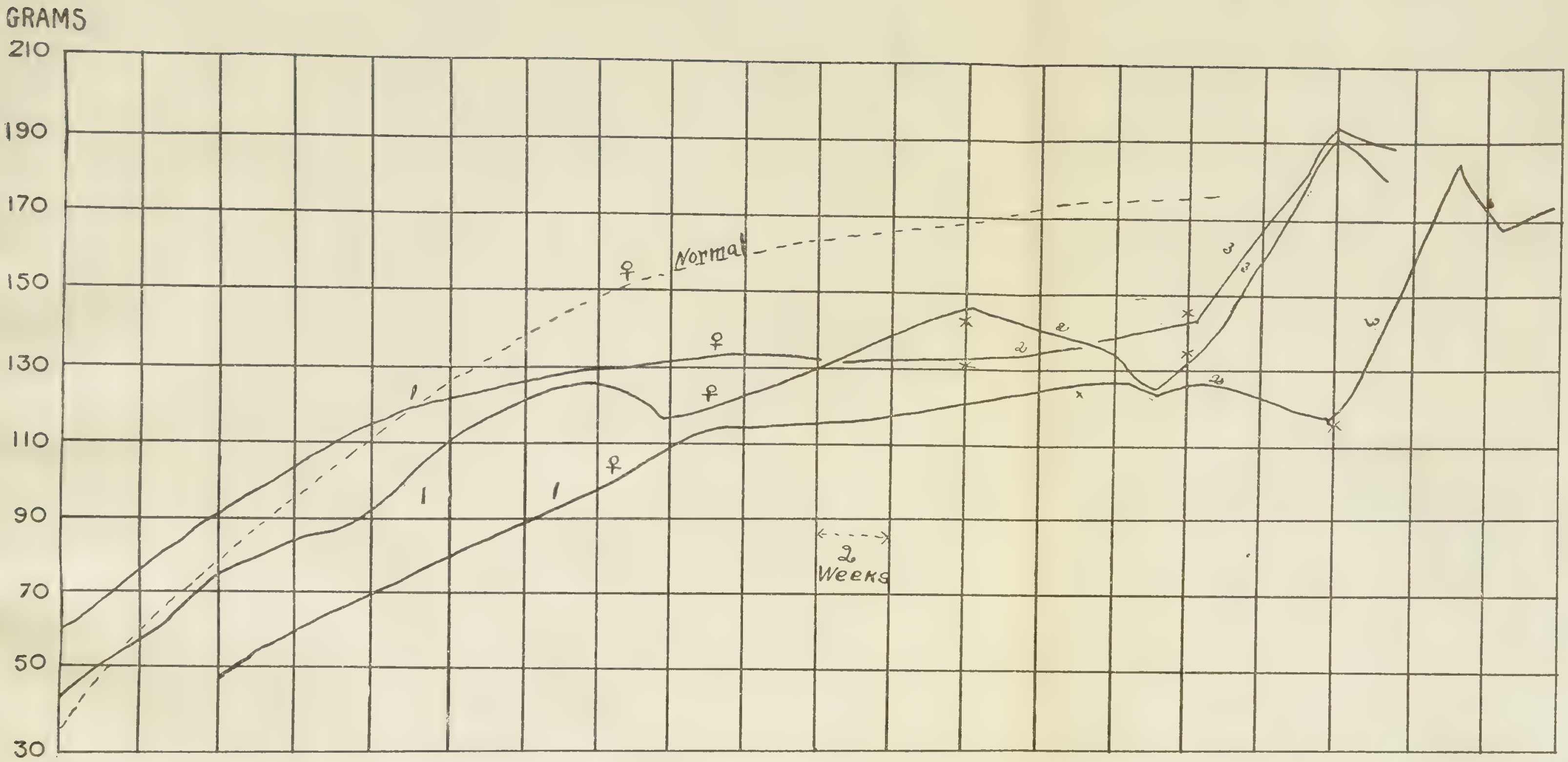
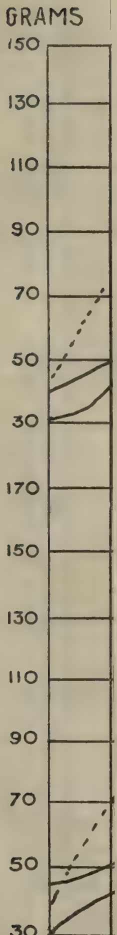


CHART VI. Furnishes comparative data as to the effects of adding 5 per cent of butter fat to the fat-free ration before failure of nutrition had set in. After the long period on the fat-free diet this was not enough butter fat to cause resumption of growth. The addition of 50 per cent corn meal to the food caused a considerable increase in body weight and improvement in appearance.

Period 1. Fat-free ration.
Period 2. Fat-free ration + 5 per cent butter fat.
Period 3. Fat-free ration 50 per cent, corn meal 50 per cent.



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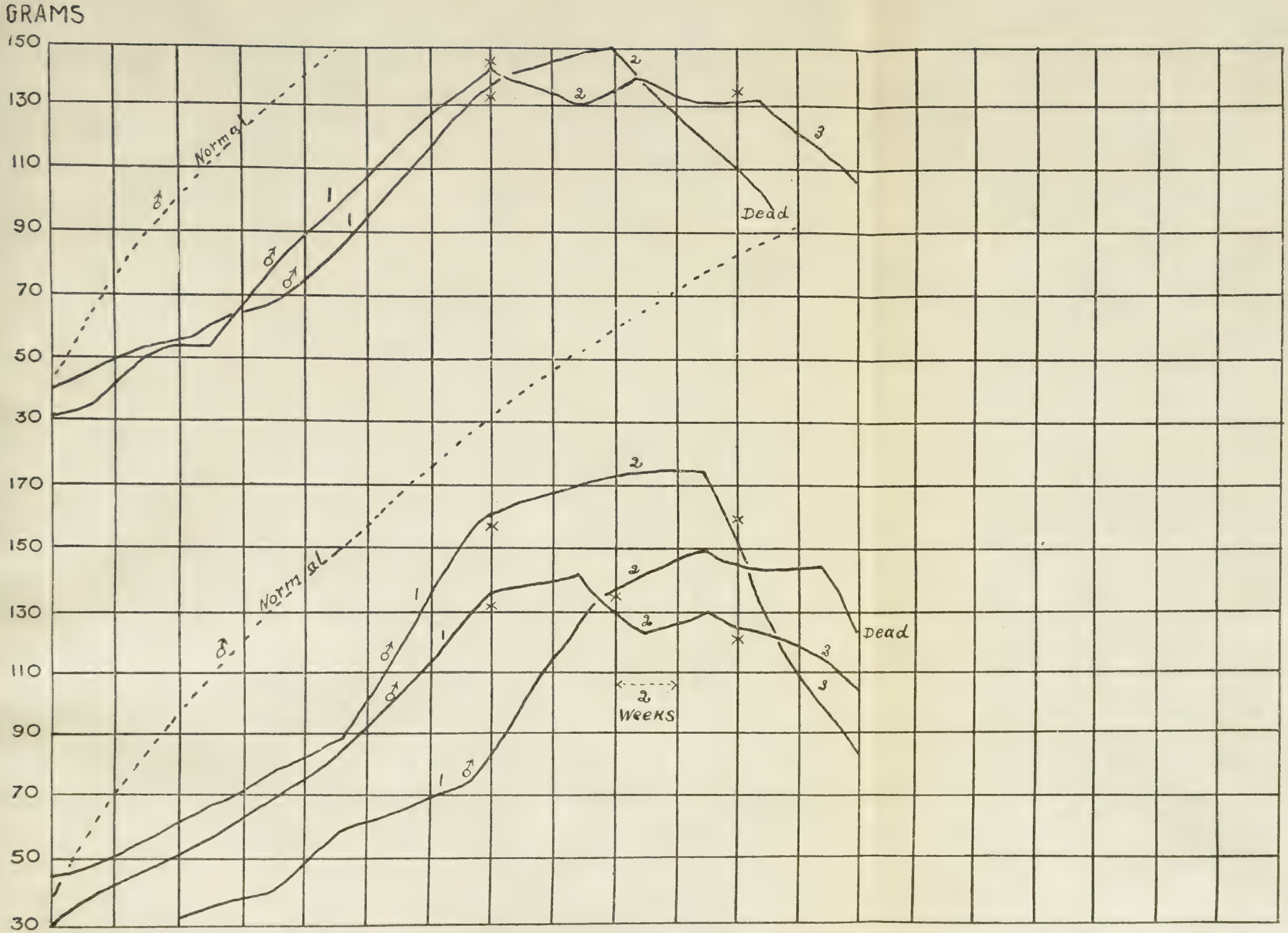
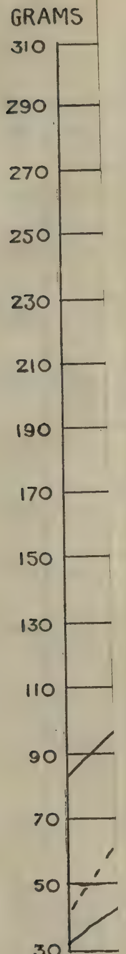


CHART VII. Furnishes comparative data relative to the amount of wheat or corn meal which is required to prevent failure on the fat-free diet. Neither 5 per cent of wheat nor corn was sufficient.

Period 1. Fat-free ration.
Period 2. Fat-free ration + 5 per cent whole wheat meal.
Period 3. Fat-free ration + 5 per cent corn meal.



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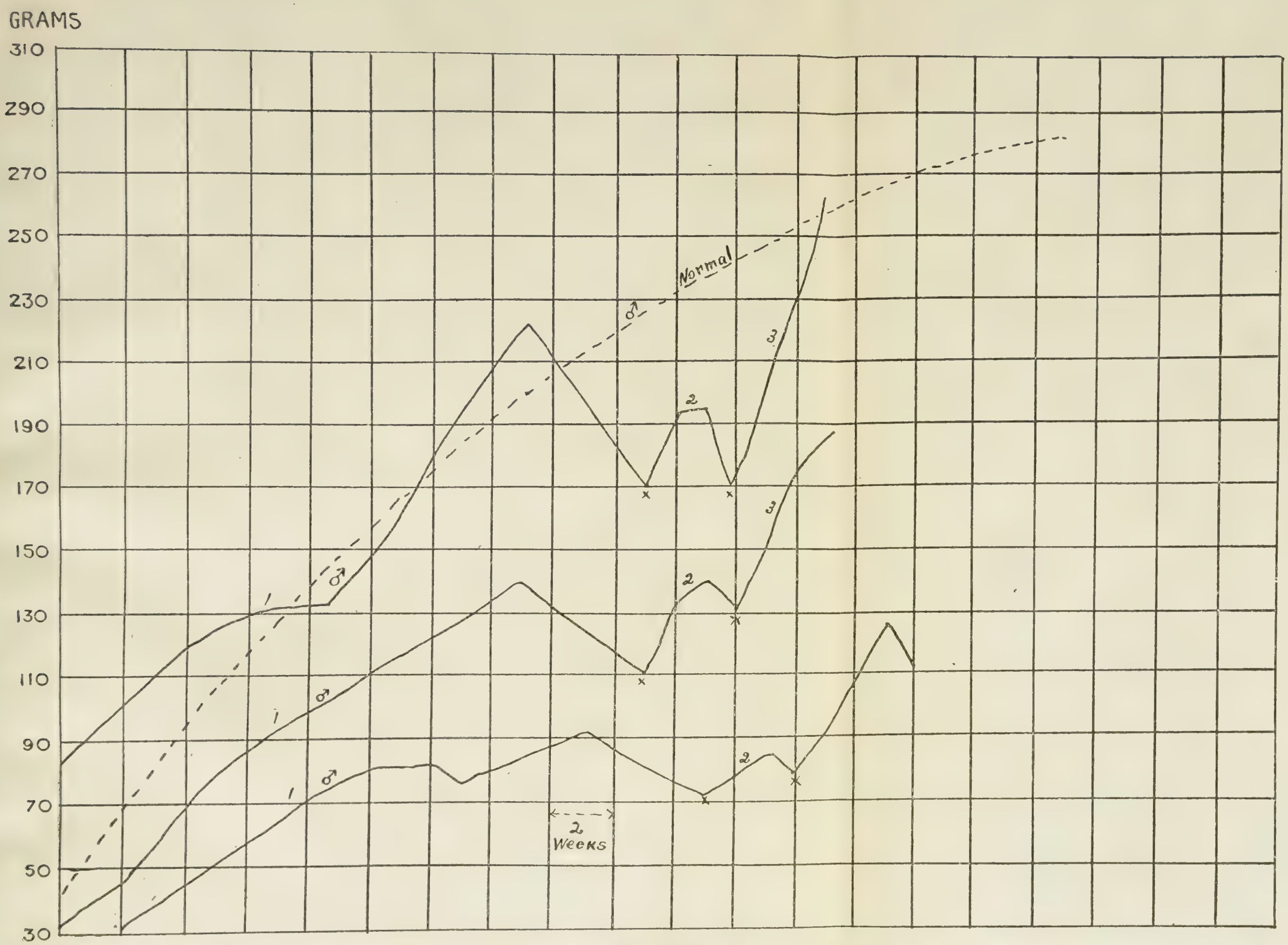

CHART VIII. Illustrates the fact that not all animal tissues will produce recovery after failure on the fat-free diet. In Period 1 the fat-free ration was fed. In Period 2 dried pig heart furnished the protein and fat of the food. In Period 3 dried pig kidney replaced the heart. The difference in the effect of these two tissues is most striking. We are not prepared to assert that the fats of the heart lack the unknown accessory substance which promotes growth. Further experiments must decide whether some other factor is involved in the heart feeding which prevents recovery.

Period 1. Fat-free ration.
Period 2. Heart 25 per cent, dextrin 74 per cent, calcium lactate 1 per cent.
Period 3. Kidney 25 per cent, dextrin 74 per cent, calcium lactate 1 per cent.

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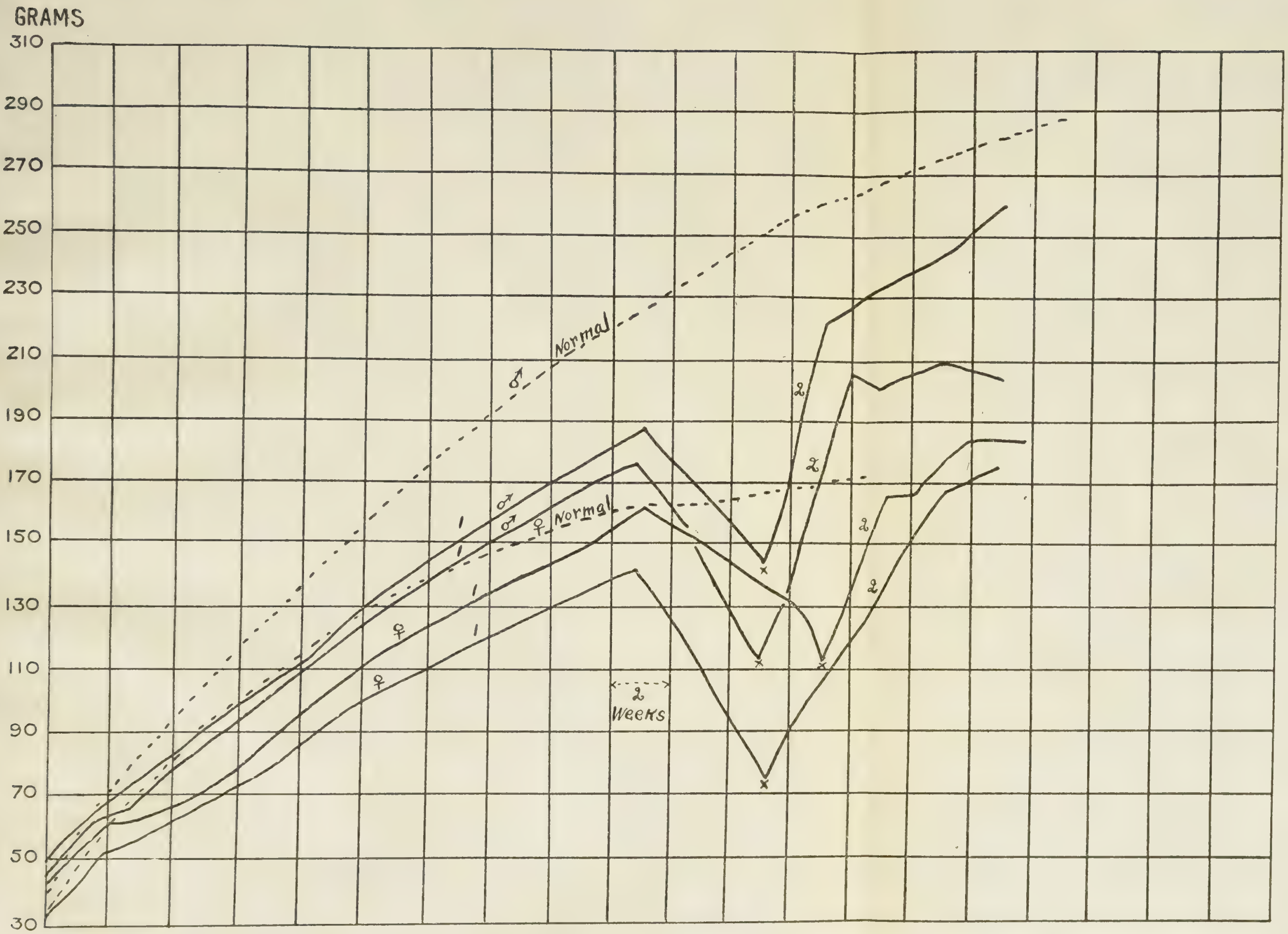

CHART IX. Illustrates the high efficiency of dried pig kidney in inducing recovery and subsequent growth in rats which were ready to die as the result of prolonged nutrition on a fat-free diet.

Period 1. Fat-free ration. Period 2. Dried pig kidney 25 per cent, dextrin 74 per cent, calcium lactate 1 per cent.

ON STARVATION AND OBESITY, WITH SPECIAL REFERENCE TO ACIDOSIS.

By OTTO FOLIN and W. DENIS.

(*From the Chemical Laboratories of the Massachusetts General Hospital and Harvard Medical School, Boston.*)

(Received for publication, March 8, 1915.)

Assuming that excreted "acetone bodies" are chiefly derived from incompletely oxidized fat, and assuming that some persons show a pronounced tendency to store up fat and become "stout," while others living under substantially identical conditions reveal no such tendency we may ask: Which, if either, of these two classes of individuals can better withstand starvation without excreting the above mentioned products of incomplete fat oxidation? Most physiologists or experts in metabolism work, if required to formulate an answer to the above, not altogether hypothetical question, would probably support the view that since fat persons tend to store fat more readily than lean ones, they probably tend to utilize it less readily, and that they therefore should be more subject to acidosis when compelled to live on their own tissue materials. Although much has been written on acidosis and still more on the metabolism of fasting there is as yet hardly any experimental work available on the basis of which any positive answer can be given to the question raised. Indeed it is only within the last few years (since the introduction of Shaffer's method for determining β-oxybutyric acid) that any satisfactory experimental material on this point could have been acquired. Brugsch's[1] investigation on the professional faster, Succi, is occasionally quoted as evidence showing that the acidosis in fasting is determined by the available fat deposits. Brugsch merely showed that the faster, Succi, who was not very emaciated at the end of his thirty day fast, continued to eliminate acetone bodies to the end of the

[1] T. Brugsch: *Ztschr. f. exper. Path. u. Therap.*, i, p. 419, 1905.

fast, while an extraordinarily emaciated hospital patient who had no visible body fat did not have any acidosis. Brugsch's results simply confirm the accepted view that the acetone bodies are derived from fat; they show nothing more, nor did Brugsch draw the conclusion ascribed to him by Sassa,[2] namely, that the extent of the fat deposit is the chief factor determining the degree of acidosis obtained in fasting. On the basis of results obtained from phlorhizinized dogs, Sassa does conclude that the extent of the fat deposits is the most important factor in the production of acidosis. Sassa's results, though interesting, have little bearing on the question of fasting acidosis in the human subject, first because of the use of phlorhizin, and secondly because there is no reason to believe that acidosis in animals is quantitatively comparable with that obtained in man. In fact the common laboratory animals (including the pig) develop very little if any acidosis during fasts of moderate duration (three to five days).

Clinically as well as from the standpoint of metabolism it is important to know whether persons with a pronounced tendency to obesity are less capable of drawing on their own fat deposits without developing acidosis than are persons without such a tendency.

In this paper we wish to report on the fasting metabolism of two extraordinarily fat women, both essentially normal except with reference to their obesity. Our analyses include practically all the determinable urinary constituents (except the metals), but our chief interest concerns the acetone bodies, and it hardly seems worth while to report all the other findings.

Our first subject (Mrs. M.), age 48, height 124 cm. (4 ft. 1 in.), entered the Massachusetts General Hospital for the relief of a large hernia which had formed in the scar of a former abdominal operation. As the surgeons advised that her weight be reduced by at least thirty-five pounds before operative procedures were instituted, the patient was transferred to the medical service and thus came under our observation.

The woman stated that she "came of a fat family," her father, mother, and brothers having all been extremely obese. She had remained quite thin as a girl, but after her marriage, at the

[2] R. Sassa: *Biochem. Ztschr.*, lix, p. 372, 1914.

age of twenty-eight, she gained rapidly in weight and had been very heavy ever since.

Our original intention was to subject her to a series of fasts of a week or more and to keep her during the interims on diets low in nitrogen and of insufficient fuel value. On account of the acidosis and the pronounced subjective symptoms (headache,

TABLE I.

Mrs. M. Urine.

DAY	ACETONE	DIACETIC ACID	β-OXYBUTYRIC ACID	NH_3N	TITRATED ACIDITY	ACETONE IN EXPIRED AIR PER HR.	REMARKS
	gm.	*gm.*	*gm.*	*gm.*	*cc.*	*mg.*	
1	0.04	0.27	0	0.41	230	0	Feeling well.
2	0.08	1.42	2.90	0.73	250	5.2	Slight headache.
3	0.10	1.57	17.94	1.87	508	24	Severe headache.
4	0.88	2.46	18.47	2.50	695	49.5	Headache, nausea, and dizziness.
15	0	0	0	0.31	180	0	Feeling well.
16	0.02	0	0	0.37	290	0	" "
17	0.03	1.17	0.17	0.53	335	30	" "
18	0.35	1.16	5.44	1.01	595	32	Slight headache, nausea.
19	0.40	1.15	13.54	1.50	655	45	Headache, nausea, and dizziness.
24	0	0	0	0.50	145	0	Feeling well.
25	0	0	0	0.37	160	0	" "
26	0.04	0.37	0.18	0.51	210	66	" "
27	0.20	1.36	17.34	0.81	300	24	Headache, nausea.

The subjective symptoms disappeared as if by magic as soon as the patient began to partake of food. A single piece of toast with a cup of coffee at once restored the patient and kept her perfectly cheerful for several hours.

nausea, and dizziness) accompanying the fasts, particularly the first one, it was deemed advisable to shorten the starvation periods.

The figures for the acetone bodies as well as for the ammonia and the acidity of the urine obtained during three successive fasting periods are given in Table I. The days are numbered so as to show the duration of the intervening feeding periods.

The elimination of the acetone bodies and the ammonia rises during the first four day fasting period to higher figures than

any heretofore recorded in connection with starvation. 10 to 12 grams of β-oxybutyric acid and not over 2 grams of ammonia nitrogen seem to represent the maximum starvation figures recorded in the literature, whereas Mrs. M. eliminated over 18 grams of β-oxybutyric acid and no less than 2.5 grams of ammonia nitrogen during her fourth day of starvation. The unusually high degree of acidosis, and the accompanying subjective symptoms thus observed in this very fat woman, seemed to indicate that persons suffering from obesity are indeed more subject to acidosis when fasting than are persons who do not carry an excessive amount of adipose tissues.

After an eleven day period on a diet which proved sufficient to cause a disappearance of the acetone bodies (in the course of three or four days), the second fast began and was continued for five days. A striking difference between the acidosis figures of this period and those of the first is to be noted. The acidosis did not begin until the third day of this fast, and the patient felt well until the fourth day, when 5.4 grams of oxybutyric acid and 1 gram of ammonia nitrogen were eliminated. On the next day (the fifth) all the subjective symptoms were severe, though the analytical results do not indicate an excessive acidosis (13.5 grams of β-oxybutyric acid and 1.5 grams NH_3N).

During the third and last fasting experiment with this patient, which began five days later, the onset of acidosis is even slower than during the second period. Indeed, the results of the three fasting periods suggest with regard to the complete oxidation of body fat in starvation that the human organism is capable of at least a certain amount of adaptation and that it is this individual factor rather than the tendency to obesity or the extent of the fat deposits in the body which chiefly determines the onset and the degree of acidosis.

In order to avoid misapprehension, attention must be called to the high β-oxybutyric acid value (17.3 gm.) and the low ammonia nitrogen (0.8 gm.) excreted on the last day of the third fasting period. The results are altogether anomalous, and it looks as if the patient had been given some alkali, a suspicion which we were, however, unable to confirm. The analyses were repeated and were found to be correct as given in the table.

If the preceding subject was fat, our next one, Mrs. B., was a veritable pork barrel. Mrs. B. entered the Massachusetts

General Hospital for the relief of a small infected abrasion of the skin on the left leg. After three or four days' treatment the lesions began to heal rapidly and gave no more trouble; and as the patient found her movements much hampered by her immense burden of fat, she readily agreed to undergo several short periods of starvation. She was 44 years old, her height

TABLE II.*

Mrs. B. Urine.

DAY	ACETONE	DIACETIC ACID	β-OXYBUTYRIC ACID	AMMONIA N	TITRATED ACIDITY	ACETONE IN EXPIRED AIR PER HR.	REMARKS
	gm.	*gm.*	*gm.*	*gm.*	*cc.*	*mg.*	
1	0	0	0	0.51	340	0	Feeling well.
2	0	0	0	0.53	365	12.6	Feeling well, but hungry.
3	0.28	0.70	0.89	0.58	330	35	Feeling well.
4	0.61	1.74	2.84	0.86	390	48	
9	0	0	0	0.52	230	0	Feeling well.
10	0	0	0	0.56	310	0	" "
11		0	0	0.68	300	16	" "
12	0.40	0.90	2.03	0.87	300	37	" "
13	0.57	2.20	7.12	1.22	295	60	Very hungry.
27	0	0	0	0.75	240	0	Feeling well.
28	0	0	0	0.45	300	0	" "
29	0	0	0	0.54	300	Trace	" "
30	0.07	0.18	0	0.57	290	28	" "
31	0.11	0.40	2.9	0.81	300	30	" "
32	1.00	1.28	7.2	1.00	380	48	Very hungry.

* The daily ration of Mrs. B. for three days preceding the first fasting period, and in the intervals between the first, second, and third fasts consisted of 200 gm. of bread, three eggs, 25 gm. of butter, and 100 gm. of boiled cabbage or spinach. No salt was allowed. Our first patient, Mrs. M., was given a somewhat similar diet. In her case, however, the food was not weighed. During the fasting periods distilled water only was taken.

was 133.5 cm. (5 ft. 4.5 in.), and she weighed 178 kilos when admitted to the hospital.

Table II contains the analytical figures bearing on the acidosis that developed in the course of three fasting experiments. On comparing these figures with those in Table I it will be seen that Mrs. B., notwithstanding her extraordinary obesity, developed nowhere near the degree of acidosis that we encountered in Mrs. M. At the end of the first four day fast the latter excreted 18.5 grams of β-oxybutyric acid in twenty-four hours, while Mrs.

B. excreted only 2.8 grams. On the fifth day of the second period the figures were 13.5 grams and 7.1 grams, respectively, and in the third period (fifth day) 13.5 as against 2.9.

In view of these results we are inclined to conclude that obesity cannot be regarded as a predisposing factor in the development of acidosis.

By comparison of the three fasting periods in Table II it will be seen that the adaptation to fasting observed in connection with the first patient is again quite unmistakable, notwithstanding the fact that the acidosis developed during the initial period was very slight indeed. We therefore concluded that one of the effects of repeated fastings is habituation to the complete oxidation of mobilized body fat, and a consequent retardation of the development of acidosis. It would, of course, have been very interesting and instructive if each of these two series of experiments could have included a great many more fasting periods, but the first patient had to be operated on, and both women were impatient to get back to their families.

The results obtained suggest, however, that one perfectly safe, rapid, and effective method of reducing the weight of very obese persons is by a series of repeated fasts of increasing duration, using the ammonia or β-oxybutyric acid elimination as a guide to the length of each fast. Qualitative tests or quantitative determinations of the acetone or the diacetic acid or both are of comparatively little value as measures of the acidosis, because whatever there is of acid intoxication must be chiefly due to the β-oxybutyric acid, and there is apparently no quantitative relationship between this acid and the other two acetone bodies.

Tables I and II also contain figures for the acetone of the expired air. The acetone of the breath has figured extensively in clinical literature on acidosis, one statement frequently encountered being that the expert clinician can detect the acetone smell as soon as he enters the room of a child or diabetic patient who has acidosis. From our quantitative figures it can be seen that the total amount of acetone eliminated through the lungs in the course of an hour always fell below 50 mg. 50 mg. per hour would amount to 1.2 grams for a full twenty-four hour day. Such an amount of acetone, and indeed several times

as much, can be scattered on the floor of a closed closet, and within an hour the odor will have disappeared.

Method used for the determination of the acetone in the breath. The patient was made to breathe through a rubber mouth-piece, such as is used in respiration work, the nose being closed by means of a nose-clip. The mouth-piece was connected with a respiration valve to which in turn was attached a wide absorption tube (inside diameter 25 mm.). The lower end of this tube was perforated by several holes (about 2 mm. in diameter), and reached to the bottom of a cylinder (60 mm. in diameter), containing 75 cc. of a 0.5 per cent solution of sodium bisulphite. The breathing

TABLE III.

Mrs. M. Urine.

DAY	VOLUME	TOTAL N	CREATININE N	CREATINE N	URIC ACID N	HIPPURIC ACID N	NaCl	WEIGHT
	cc.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*kg.*
1	495	6.8	0.33	0.0	0.09	0.04	0.67	108.2
2	550	6.8	0.33	0.06	0.08	0.03	0.40	
3	1130	8.7	0.35	0.12	0.08	0.03	0.32	
4	1140	9.4	0.35	0.18	0.12	0.03	0.31	104.7
15	340	3.1	0.30	0.0	0.07	0.04	0.14	104.0
16	345	3.1	0.33	0.0	0.07	0.02	0.16	
17	420	4.3	0.33	0.03	0.07	0.02	0.17	
18	580	5.5	0.33	0.03	0.07	0.02	0.16	
19	750	5.2	0.33	0.01	0.07	0.02	0.15	100.9
24	450	5.5	0.33	0.05	0.09	0.05	0.18	100.5
25	400	4.0	0.33	0.03	0.08	0.03	0.20	
26	370	4.7	0.31	0.02	0.08	0.02	0.17	
27	560	4.5	0.33	0.02	0.07	0.02	0.19	98.9

through this absorption apparatus was continued for one to two minutes.[3] The solution was then made up to a definite volume and the acetone was determined in an aliquot part by our turbidity method.

In Tables III and IV are given a few additional analytical data which it has seemed best to put on record.

Creatinine and creatine. The creatinine figures are given partly as an index of the degree of completeness of the urine

[3] Both of our patients had been used many times in respiration experiments in connection with the determination of basal metabolism and had been trained to maintain their normal respiration rate when breathing through the apparatus.

collection, although the quantitative collection of urine from fasting women is a relatively simple matter, since defecation occurs so seldom in this condition. Except during the first period with our second patient the creatinine elimination remained very constant.

The creatinine figures indicate one other point which is not without interest. Our second patient has been presented as being, if anything, fatter than the first, their weights being 178 and 108 kilos, respectively, at the beginning of the first fast;

TABLE IV.

Mrs. B. Urine.

DAY	VOLUME	TOTAL N	CREATININE N	CREATINE N	URIC ACID N	NaCl	WEIGHT
	cc.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*kg.*
1	540	9.1	0.74	0.10	0.10	0.54	178
2	600	10.1	0.74	0.10	0.12	0.54	
3	420	8.0	0.60	0.12	0.13	0.38	
4	500	8.0	0.49	0.25	0.12	0.38	175
9	400	5.2	0.65	0	0.14	0.30	174.4
10	310	5.5	0.66	0	0.14	0.21	
11	400	7.1	0.66	Trace	0.13	0.26	
12	390	6.7	0.66	0	0.12	0.21	
13	520	8.3	0.64	0	0.12	0.26	170.5
27	320	5.8	0.65	0	0.12	0.14	168.2
28	400	6.2	0.65	0	0.12	0.12	
29	355	7.0	0.65	Trace	0.12	0.14	
30	420	6.3	0.65	0	0.11	0.14	
31	390	6.7	0.72	0	0.11	0.14	
32	480	6.8	0.72	0	0.12	0.14	164.6

judging by the weights, appearance, and body measurements that seemed to be true.

From the acidosis standpoint, both patients were certainly extremely obese, without regard to which may have carried the greater amount of inert fat per unit of fat-free living tissue. The creatinine figures indicate perhaps that the less heavy woman, Mrs. M., was the more obese of the two, since she excreted only half as much creatinine per day as Mrs. B., yet weighed considerably more than half as much as the latter. The "creatinine coefficient" is, however, very variable in women, and it is doubt-

ful how much significance can be attached to the creatinine elimination as an index of obesity. The muscular tonus of Mrs. M. was probably greater than in Mrs. B. Except when fasting the former was very active and carried her weight well, while the latter declined to take any exercise whatever.

We have included our creatine figures in the tables, although we do not care to discuss them at the present time. Every precaution was taken to remove the acetone and diacetic acid before making the creatinine determinations. The figures given for the creatine nitrogen are, therefore, of some interest in connection with the conclusion of Graham and Poulton, that the so called creatine in urine of fasting subjects represents nothing else than errors in the determination of the preformed creatinine, and that no creatine is obtained if the diacetic acid is destroyed before determining the preformed creatinine.

Total nitrogen and uric acid. The total nitrogen elimination is strikingly low in both subjects. The current opinion that fasting adults reach an approximately common and relatively high level of nitrogen elimination (10 to 14 grams) after three to five days of fasting, and this independently of their previous diet, is probably not correct. In this connection we might cite the figures which one of us obtained several years ago from a man who began to fast at the end of a two week feeding experiment on an almost protein-free ("starch and cream") diet.

	1st day	2d day	3d day	4th day	5th day
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
Nitrogen	4.4	6.5	10.8	11.0	12.2
Uric acid N	0.15	0.21	0.22	0.21	0.23

This subject was neither fat nor unusually emaciated and was confined to his bed during the starvation period. His daily creatinine N elimination was 0.33 gram; *i. e.*, exactly the same as that given by Mrs. M.

Two conclusions with reference to the nitrogen elimination suggest themselves: first, that the obese destroy less body protein during moderate periods of starvation than others; and, secondly, that with repeated fastings their adaptation to the complete utilization of body fat, which was indicated by a re-

tarded and diminished excretion of acetone bodies, is also accompanied by a sparing of the body protein. The latter conclusion may be regarded as a verification of a similar deduction arrived at by Howe and Hawk[4] in their experiments on dogs.

The uric acid output is also remarkably small in our obese women as compared with the uric acid values obtained from the man. Opinions differ as to whether the uric acid elimination in man is or is not completely independent of the total nitrogen elimination. Since the metabolism of glandular organs plays a much greater rôle in the production of purines than in the setting free of creatinine there is no reason why the total protein metabolism might not affect the total purine production more than it affects the production of creatinine, and our results indicate that such is the case.

SUMMARY.

Obesity is not a predisposing or contributing factor in the onset or intensity of the acidosis of starvation.

The total acetone excretion with the breath in starvation is quantitatively insignificant (at most 1 gram per day), and the notion, current among clinicians that they can smell acetone "all over the room" when a case of acidosis is present, is erroneous.

By repeated fasts of moderate duration the obese acquire an increased ability to starve without the production of acetone bodies.

The obese lose less body protein than others in the course of moderate periods of starvation (four to six days), and on repeating the fasts the losses of body protein become still smaller.

Successive moderate periods of starvation constitute a perfectly safe, harmless, and effective method for reducing the weight of those suffering from obesity.

[4] P. E. Howe and P. B. Hawk: *Jour. Am. Chem. Soc.*, xxxiii, p. 253, 1911.

NOTE ON PERCA GLOBULIN.

By OTTO FOLIN and W. DENIS.

(From the Biochemical Laboratory of Harvard Medical School, Boston.)

(Received for publication, March 8, 1915.)

In 1903 Mörner[1] accidentally discovered a peculiar protein substance in the eggs of the ordinary perch (*Perca fluviatilis* L.) to which he gave the name perca globulin. The substance was particularly abundant in the viscous fluid surrounding the eggs. It gave the reactions of globulins, but its chief characteristic is a pronounced astringent taste which differentiates it from all other protein materials found in the eggs of other species of fish. It is further peculiar in that it gives precipitates with ovomucoid and other glycoproteids, and is precipitated by fairly concentrated hydrochloric acid solutions (0.75 per cent).

Having been unable to find a similar substance in the roe of any other fish, not even in the sea perch (*Labrax lupus* Cuv.), Mörner asked us some time ago to examine the eggs of the American perches in order to determine whether these also contain the unusual globulin in question.

Through the courtesy of H. M. Smith of the U. S. Bureau of Fisheries we obtained last spring samples of ripe and unripe roe from yellow perch and pike perch. The aqueous extracts from these were tested with hydrochloric acid and with ovomucoid, according to Mörner's directions. The available samples were rather small, and with our dilute extracts the precipitates obtained with the above mentioned reagents were not very strong. The hydrochloric acid precipitates dissolved when we added enough hydrochloric acid to give a concentration of 0.75 per cent. The peculiar astringent taste reported by Mörner, was, however, pronounced and unmistakable in all of the extracts. In the unripe eggs it was much more pronounced than in the ripe ones.

[1] C. T. Mörner: *Ztschr. f. physiol. Chem.*, xl, p. 429, 1903.

The precipitates obtained with hydrochloric acid and with ovomucoid seemed to correspond approximately to the intensity of the astringent taste.

We conclude therefore that the perca globulin discovered by Mörner in the common European perch (*Perca fluviatilis* L.) is also present in the roe of the two American perches, *Perca flavescens* and *Perca sorzoscedion* (yellow perch and pike perch).

NOTE IN DEFENSE OF THE FOLIN-FARMER METHOD FOR THE DETERMINATION OF NITROGEN.

By OTTO FOLIN.

(*From the Biochemical Laboratory of Harvard Medical School, Boston.*)

(Received for publication, March 13, 1915.)

In the January number of this Journal Bock and Benedict criticise the Folin-Farmer colorimetric method for determining nitrogen in urine, and describe two modifications which they find give better, though still unsatisfactory results. The relative merits of the modifications may be left out of consideration, for the present at least; because preferences in such matters are determined more by the facilities available in different laboratories than by the comparative accuracy of which each process is capable.

The criticisms of the original method I cannot accept in silence inasmuch as scores of students and many mature persons are every year learning the method in our laboratories. Within the last few weeks I have had two such, each of whom had, however, to spend about two weeks before satisfactory agreement between the results of the colorimetric and of the Kjeldahl method was obtained.

The first thing that students do in learning the method is to take a standard ammonium sulphate solution and determine the ammonia in it by the aeration-colorimetric process, using the same ammonium sulphate solution as the standard. If they get too low results they are told to check up the completeness of the aeration by means of an additional aeration leading the air into a second receiver containing distilled water acidified with hydrochloric acid and to test for ammonia with Nessler's reagent. They thus get acquainted with the air current, and incidentally demonstrate that the air contains no ammonia.

This preliminary work also proves to their satisfaction and mine that ammonia can be determined by means of the aeration-

colorimetric method to the same degree of accuracy as is obtained in reading the standard solution against itself. The next step in learning the process is to apply the micro Kjeldahl treatment to the standard ammonium sulphate solution and again determine the ammonia. This process is repeated until consistently satisfactory results are obtained.

The suggestion of Bock and Benedict that there are several inherent errors in the method, errors amounting to 5 to 10 per cent, errors which are sometimes additive and sometimes (nearly) balance each other, sounds more antagonistic than convincing. Such antagonism toward a new process offered in place of the tried and reliable Kjeldahl process is, of course, quite legitimate. The Kjeldahl method had to pass through many years of unfavorable comparisons with the older and reliable Dumas method, and it took scores if not hundreds of researches before the merits and the limitations of the Kjeldahl method became a matter of generally accepted knowledge. In fact, the agricultural chemists are still publishing official reports on various forms of the method.

Bock and Benedict find that when they pass ammonia-free air into acidified water and Nesslerize, traces of ammonia are obtained. I have already explained that this phenomenon does not happen in our laboratories, and the point is checked up by every careful new learner of the method. That it does not occur here can readily be inferred from the discussion by Folin and Macallum on the determination of ammonia in urine.[1] Bock and Benedict explain the finding of ammonia in blank tests on the basis of back diffusion resulting in the absorption of ammonia from the laboratory air. The explanation seems rather fanciful and cannot be correct, since it is not encountered here. A more plausible explanation is perhaps to be found in the fact that the air supply in Benedict's laboratory does not come from out of doors. Our air supply does, and with us it makes no demonstrable difference whether the air used in driving over the ammonia is or is not washed with sulphuric acid, though we always do first pass the air through dilute sulphuric acid contained in two liter bottles. We use these wash bottles less because of the ammonia

[1] O. Folin and A. B. Macallum: this *Journal*, xi, p. 524, 1912.

than because of the moisture of the compressed air which sometimes condenses in the pipes.

As a matter of curiosity Dr. Denis and I determined a short time ago the ammonia present in three wash bottles which had been used without change in my laboratory since November 16, 1911; *i.e.*, about three and a half years. Hundreds of aerations, many lasting for hours, must have been made through each, and back diffusion through open rubber tubes could have occurred during the entire period. Originally each bottle contained 500 cc. of water and 50 cc. of concentrated sulphuric acid. The total ammonia nitrogen found in the bottles amounted in each case to 8 to 10 mg.

I confess to some skepticism concerning the ammonia obtained by Bock and Benedict in their blank experiments. With my older aeration method for the determination of ammonia, the air current in many laboratories was used for several hours and blank errors such as are quoted by Bock and Benedict could hardly have escaped detection. It seems to me more probable that the "ammonia" obtained from the laboratory air by Bock and Benedict represents reducing substances, such as aldehyde, hydrogen sulphide, etc. The finding is nevertheless of some importance, of course, because it evidently represents a possible source of error in colorimetric ammonia determinations when inside laboratory air is used.[2] The error due to such sources is, however, not comparable to the error due to ammonia. In the case of the latter the error would be approximately proportionate to the time the air current is used, whereas with volatile reducing bodies there would probably be obtained as much apparent ammonia at the end of two minutes as at the end of two hours. Bock and Benedict have recorded only the results of ten minute periods.

In connection with their discussion of ammonia absorption from the laboratory air, Bock and Benedict take occasion to express grave doubts as to the validity of the ammonia determinations in blood published by

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[2] If the results reported by Bock and Benedict had come from a less competent investigator than Benedict, I should have suspected dirty utensils as the source of the ammonia, because of the prevalence of ammonium salt deposits in chemical laboratories.

Folin and Denis. We have never attached any great significance to the absolute figures for the ammonia found in the general systemic blood, because the values are so extraordinarily small. An error of 50 to 100 per cent in the figures is practically immaterial. As compared with results obtained by any other known method we believe that ours are the most reliable. Since the doubts expressed by Bock and Benedict are based on observations which have no bearing on our work and on interpretations which I believe to be erroneous, further defense of the figures published by Folin and Denis is, I think, not necessary.

Other sources of possible error suggested by Bock and Benedict are: (1) traces of ammonia in the sulphuric acid and potassium sulphate,—an error which applies with nearly equal force to the Kjeldahl method; and (2) unavoidable errors in measuring 1 cc. with Ostwald pipettes and in the readings of the colorimeter.

The error in the use of Ostwald 1 cc. pipettes Bock and Benedict estimate at 1 per cent or over, whereas Farmer and I have explicitly stated that when used as described the error is only about 0.1 of 1 per cent.[3] Our method of using the pipettes represents no unusual or impracticable refinements, and the ten-fold multiplication of this error indulged in by Bock and Benedict I regard as unwarranted and unfair. The point involved may seem small to the casual reader, but the whole system of analysis which has been developed and is being further developed in this laboratory depends on the use of these pipettes, and I cannot allow such criticism to pass without a protest. The other day I took at random one of a dozen pipettes and with it measured into four 100 cc. Erlenmeyer flasks 1 cc. of normal hydrochloric acid. To each I added 25 cc. of water and four drops of indicator ("methyl red"). Then I prepared some approximately 0.025 normal sodic hydrate and titrated the contents of the flasks to the same end-point. The following figures were obtained: 42.1, 42.1, 42.1, 42.05, expressed in cc.

The accuracy of the colorimetric readings is unfortunately more subject to the "personal equation," and it doubtless does amount to 1 per cent as Bock and Benedict state, at least in many cases. Using a clean colorimeter and setting both sides of the instrument at 20 mm., with the standard solution in both and thus getting the appearance of the theoretical equality fixed, I find

[3] O. Folin and C. J. Farmer: this *Journal*, xi, p. 494, 1912.

that not only I, but the majority of other workers, can make the subsequent readings practically perfect. Experience in the use of the colorimeter counts for much, and here where it is constantly used for many different kinds of determinations the errors due to uncertainties in the readings average, I think, considerably less than 1 per cent.

Those who cannot readily learn to use the colorimeter with accuracy and certainty can, of course, substitute titration with 0.02 N sodic hydrate.

"Methyl red" appears to be much superior to "alizarin red" which we formerly recommended as indicator in connection with ammonia titrations.

As against the formidable array of analyses offered by Bock and Benedict in proof of their conclusion that our method gives results which vary from +4 to −11 per cent from the values obtained by the Kjeldahl method, I can only refer to the figures published by Farmer and myself.[4] I made all the colorimetric readings involved in those determinations and did not know the values given by the Kjeldahl process until after my readings had been recorded.

It is to be noted that in 57 of the 71 analyses reported by Bock and Benedict the errors recorded represent *losses* of ammonia. If the source of those losses had been located and corrected their analyses would have had a very different appearance and the described modifications would have appeared less unmistakably superior to the original method. Bock and Benedict could, of course, not rectify the error since their air supply yielded "ammonia" even after being washed by 50 per cent sulphuric acid. When I drive 1 mg. of ammonia nitrogen into a 100 cc. volumetric flask containing 30 to 50 cc. of water and 2 cc. of 0.1 N HCl in such a way that all of it goes over in ten minutes, and pass the air current coming from the flask into a test-tube containing 2 to 3 cc. of acidified water, I find from 0.0005 to 0.003 mg. of ammonia nitrogen in the latter; *i.e.*, a loss of from 0.05 to 0.3 of 1 per cent.

[4] Folin and Farmer: *loc. cit.*, p. 501.

CREATININE AND CREATINE DETERMINATIONS.[1]

THE OCCURRENCE OF CREATINE.

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(Received for publication, March 24, 1915.)

Interference with the usual colorimetric procedures for determining creatinine and creatine in the urine of diabetics and of those undergoing starvation has long been attributed to the presence of acetone bodies and glucose. The conversion of glucose and other sugars to interfering acids during the hydrolysis of creatine introduces an error which makes the creatinine-creatine value questionable. The presence of acetone bodies in the various possible proportions renders the quantities of creatinine and creatine both uncertain, since the latter is determined as an addition to the former. Whether the effect is an apparent increase in creatinine and a corresponding decrease in creatine value, or the reverse, is a question with evidence on each side. Klercker[2] and Graham and Poulton[3] agree that acetone causes a decrease in the creatinine results, while Van Hoogenhuyze and Verploegh[4] and Krause[5] report a temporary elevation, but later disappearance of the excess color, allowing the value to become correct. Krause, Wolf and Osterberg,[6] Rose,[7] and Graham and Poulton[8] found that aceto-acetic acid, or its ester, increases the creatinine

[1] A preliminary report of the methods here published was made before the Society of Biological Chemists, December 28, 1914.

[2] K. O. Klercker: *Biochem. Ztschr.*, iii, p. 45, 1907.

[3] G. Graham and E. P. Poulton: *Proc. Roy. Soc., Series B*, lxxxvii, p. 205, 1913–14.

[4] C. J. C. Van Hoogenhuyze and Verploegh: *Ztschr. f. physiol. Chem.*, lvii, p. 161, 1908.

[5] R. A. Krause: *Quart. Jour. Exper. Physiol.*, iii, p. 289, 1910.

[6] C. G. L. Wolf and E. Osterberg: *Am. Jour. Physiol.*, xxviii, p. 71, 1911.

[7] W. C. Rose: this *Journal*, xii, p. 73, 1912.

[8] Graham and Poulton: *loc. cit.*

value, though the two last authors found the opposite effect due to *small* amounts of the ester. Graham and Poulton further report that sodium aceto-acetate decreases the apparent value of creatinine. Conflicting as these results appear, the conclusion must be drawn that there is an undoubted interference due to the presence of acetone and aceto-acetic acid (the effect of β-oxybutyric acid is considered negligible), though its exact nature may depend upon factors as yet undetermined.

When the presence of these substances is known, their removal before proceeding with the determinations has not proved easy. Greenwald[9] suggested an extraction-aeration method for freeing urine of aceto-acetic acid. This required three hours; and Graham and Poulton[10] proposed to substitute distillation with phosphoric acid under reduced pressure. Lately[11] these two workers have returned to the ether extraction method, but increase the extraction time to twelve hours. The possibility of shortening the time, or of simplifying the process by which the interference can be avoided, prompted the work here reported.

In previous investigations[12] which included creatinine and creatine determinations in rat urine, I had used standard solutions of the double picrate of potassium and creatinine. In preparing this interesting double salt from urine it was found to precipitate almost quantitatively. Whereas Jaffe[13] had found the solubility of this salt in water to be a little less than 0.2 gram in 100 cc., I found its solubility in urine saturated with picric acid to be about one-fourth that amount. If conditions could be maintained, it seemed that this salt offered a means of separation from the disturbing factors. The method to be described accomplishes such a separation (with a fairly constant amount remaining in solution). In addition to maintaining conditions of precipitation it is only required to redissolve the double salt and determine the creatinine value of this solution by the modified Folin[14] method.

[9] I. Greenwald: this *Journal*, xiv, p. 87, 1913.

[10] Graham and Poulton: *loc. cit.*

[11] Graham and Poulton: *Jour. Physiol.* (*Proc. Physiol. Soc.*, lv), xlviii, 1914.

[12] O. Folin and J. L. Morris: this *Journal*, xiv, p. 509, 1913.

[13] M. Jaffe: *Ztschr. f. physiol. Chem.*, x, p. 397, 1886.

[14] Folin and Morris: this *Journal*, xvii, p. 469, 1914.

The details of the method are as follows: To 100 cc. of urine in a beaker add 1 gram of solid picric acid; warm to hasten the solution of the picric acid, but heat on a water bath to temperatures not higher than 60°C. Allow the mixture to cool, stir well, and at the expiration of four hours the creatinine is all precipitated except a fairly constant amount which remains permanently in solution. (There is no additional precipitation after two days.) Now decant through a Gooch crucible and return the portion of the precipitate thus caught on the asbestos mat to the beaker. Dissolve the double salt by heating on a water bath with 100 cc. of approximately $\frac{N}{2}$ HCl and add hot rinse waters to make up to volume in a 500 cc. flask. Filter a portion from asbestos and determine the creatinine in a 10 cc., 5 cc., or smaller portion of the solution, according to which volume gives a value nearest 1 mg. standard. The standard I have preferably used in all creatinine determinations is a solution of potassium creatinine picrate of such strength that 10 cc. contain 1 mg. of creatinine (0.5388 gram of double salt in 1000 cc. $\frac{N}{10}$ HCl). This strength standard is very advantageous in that it is available alike for determining creatinine values of solutions containing 0.1 mg. per 1 cc., and those containing ten or fifteen times that amount. When solutions of the latter strength are being determined the 1 cc. portions taken for analysis are diluted to 10 cc. before the reagents are added, thus maintaining the same percentage alkalinity in all the reaction mixtures.

The determination of creatine was carried out in a very similar manner. Conversion of creatine to creatinine is accomplished by the Myers[15] autoclave method. I use 100 cc. of urine and add 20 cc. of 5 N HCl to it. At the expiration of about forty minutes the urine is removed from the autoclave and 10 per cent NaOH added to neutralize the HCl. The end-point desired is slightly acid, with organic acids (red to Congo, but red also to litmus). A heavy precipitate will appear. Make up to 200 cc., filter, and collect 100 cc. for precipitation as in the creatinine determination. The conditions of precipitation alone vary, 1.5 gram of picric acid being used and the solution hastened by heating over a free flame. The precipitation is somewhat slower, and

[15] F. G. Benedict and V. C. Myers: *Am. Jour. Physiol.*, xviii, p. 397, 1907.

I usually leave it over night. In calculating, allowance must be made for the fact that 100 cc., from which the creatinine-creatine (total creatinine) is precipitated, correspond to half that volume of the original urine.

The precipitation is not complete in either case. The extreme variations of the amount remaining in solution are from 5 mg. in 100 cc. of a pure aqueous solution of creatinine (with only a little KH_2PO_4 added to supply the potassium for the double salt) to 15 mg. in the most concentrated solution analyzed for creatinine-creatine. An adjustment for this incomplete precipitation, which has proven satisfactory, is made by adding an average correction of 8 mg. in each determination of creatinine and an average correction of 12 mg. in each determination of creatinine-creatine. These figures are used on the basis of the uniform volume of 100 cc. of solution precipitated, and have been added to all results of the precipitation method appearing in the tables. The size of these corrections, as well as the greater time required for an analysis, renders the method less desirable for general routine use than the usual procedures of Folin[16] and Myers.[17] However, in cases of evident interference, the method is a much easier one than those of extraction and distillation. That it efficiently does away with these troubles appears in the results recorded in Tables I and II.

Table II presents comparative results of creatinine and creatinine-creatine determinations made on the same urines by the use of the Folin, Myers, Benedict,[18] and precipitation methods. The urines there reported include one from a diabetes case, one from a patient undergoing starvation, and two normal urines with large amounts of sodium aceto-acetate, acetone, and glucose added. These results and many other similar ones, seem to indicate the nature of the interference in the Folin, Myers, and Benedict methods of analysis due to the presence of acetone bodies and glucose. As interpreted on the basis of the precipitation method, there is an apparent creatinine value given by the Folin method in the presence of acetone bodies, which is less than the true value. This decrease very gradually disappears, allowing

[16] Folin and Morris: *loc. cit.*

[17] Benedict and Myers: *loc. cit.*

[18] S. R. Benedict: this *Journal*, xviii, p. 191, 1914.

TABLE I.

Creatinine and creatinine-creatine determinations in normal urine with and without the addition of creatine, glucose, and acetone bodies.

Addition in gm. per 100 cc.		Urine 1	Urine 2-A	Urine 2-B	Urine 3
*Creatine		0	0.076	0.076	0.038
Glucose		0	0	5	0
Acetone bodies		0	0	0	β-Oxybutyric acid 1 gm., aceto-acetic ester 1 gm., acetone 0.5 gm.
Creatinine	Precipitation method	0.138	0.132	0.130	0.119
	Folin method (revised)	0.136	0.130	0.127	0.108 to 0.121 (during 3 hrs.)
Creatinine-creatine	Precipitation method	0.137	0.205	0.203	0.157
	Folin method (revised)	0.139	0.207	0.211	0.162
	Myers method	0.141		0.220	0.174
	Benedict method	0.148		0.228	0.164

All figures are in gm. per 100 cc. of volume.
*These figures represent the creatinine equivalent of the creatine added.

the reading to approach the true one after two or three hours. In the presence of glucose, however, the increase is supplemented by another, due probably to the picramic acid[19] formed, and the increase continues far beyond the true value of the creatinine. Further, the status of the creatinine-creatine determination seems to be that the Folin method gives about the true value, avoiding practically all interference, while both the Myers and Benedict methods give high values, due largely to the decomposition products of glucose.

[19] R. C. Lewis and S. R. Benedict: *ibid.*, xx, p. 61, 1915.

TABLE II.

Comparative creatinine and creatinine-creatine results determined by several methods in urines containing interfering substances. The lower figures in the first and second columns are the corresponding twenty-four hour quantities. The lower figures in the third and fourth columns are the theoretical figures per 100 cc.

		DIABETIC* URINE	STARVATION** URINE	URINE A*** (Aceto-acetic acid, acetone and glucose added to each. Compare note)	URINE B****
Creatinine	Precipitation method	0.079	0.039	0.118	0.092
		0.99	0.98		0.095
	Folin method (revised)	0.069 to 0.075 (during 5 hrs.)	0.031 to 0.034 (during 2 hrs.)	0.104 to 0.125 (during 3 hrs.) 0.144 at end of 6 hrs.	0.076 to 0.127 (during 2½ hrs.) 0.323 at end of 15 hrs.
		0.86–0.94	0.78–0.86		
Creatinine-creatine	Precipitation method	0.130	0.056	0.161	0.168
		1.63	1.41	0.156	0.171
	Folin method (revised)	0.140	0.057	0.167	0.176
		1.75	1.44	0.156	0.171
	Myers method	0.151	0.059	0.182	0.193
		1.89	1.49	0.156	0.171
	Benedict method		0.057	0.175	0.206
			1.44	0.156	0.171

*B. W., 14 yrs. old, on carbohydrate-free diet; acidosis and small amount of glucose.

**McC., first day on Lenhartz diet, following 2 weeks' starvation; acetone and aceto-acetic acid 1.65 gm., β-oxybutyric acid 5.0 gm. in 24 hrs.

***A., artificial diabetic urine; 1000 cc. contained 100 cc. of mixture of sodium aceto-acetate and acetone (made by treating aceto-acetic ester with NaOH for 36 hrs.), 30 gm. glucose and 0.500 gm. creatine (equivalent to 0.38 gm. creatinine), and urine to make liter.

****B., artificial diabetic urine; 1000 cc. contained 200 cc. of sodium aceto-acetate and acetone mixture, 50 gm. glucose, 1.000 gm. creatine (equivalent to 0.760 gm. creatinine), 700 cc. urine of creatinine, value 0.136 per 100 cc., and water to make up to volume. $7/10 \times 0.136 = 0.095$ gm. calculated value of 100 cc. of urine as prepared.

The question sometimes has arisen whether the Jaffe reaction really is a measure of the creatinine present, to the exclusion of other substances which give the same or similar color with the alkaline picrate. The precipitation of creatinine as the double picrate is so specific a reaction, no appreciable amount of creatine

TABLE III.

Occurrence of creatine in urine.

		CHILD	POST PARTUM (3D DAY)	DIABETIC	STARVATION
Creatinine	Precipitation method	0.083	0.151	0.135	0.033
		0.62	1.07	1.42	0.79
	Folin method (revised)	0.081	0.151	0.133	0.028 to 0.030 (during 4 hrs.)
		0.61	1.07	1.40	0.67 – 0.72
Creatinine-creatine	Precipitation method	0.101	0.232	0.167	0.048
		0.76	1.65	1.75	1.10
	Folin method (revised)	0.098	0.227	0.165	0.046
		0.74	1.61	1.73	1.10
	Myers method	0.101	—	0.177	0.047
		0.76	—	1.86	1.13
	Benedict method	0.091	0.227	—	—
		0.68	1.61	—	—

The upper figures represent gm. per 100 cc.; the lower figures, gm. per 24 hrs.

being precipitated though added in large quantity to urine (compare Table I), that this method of its determination may serve for identification wherever doubt may arise. Further, the precipitation method for determining creatinine-creatine will answer the related question as to whether the 'creatine' appearing as an additional amount of creatinine after hydrolysis is really such, or some other substance or substances converted into a form giv-

ing the color reaction. Precipitation as double picrate of this resultant 'creatinine' under the same quantitative conditions as with creatinine would seem to offer a final answer to this doubt. The results appearing in Table III conclusively prove that the often reported creatine in the urine of children, women in post partum, diabetics, and starvation cases is actually converted quantitatively into creatinine by heating in acid solution, and can be obtained as double picrate according to all the conditions of precipitating creatinine.

CONCLUSIONS.

1. Precipitation of creatinine as creatinine potassium picrate is offered as a means of freeing the colorimetric determination of creatinine and creatine from the interfering presence of acetone bodies and glucose.

2. Comparative results obtained by this precipitation method and the Folin, Myers, and Benedict methods upon the same urines indicate the following effects of acetone bodies and glucose upon the true creatinine and creatine values: (a) The Folin method for creatinine gives an immediate decrease due to acetone bodies, followed by a very gradual increase to a much exaggerated level, due to glucose. The Folin method for creatinine-creatine suffers little or no interference, but the decrease in creatinine correspondingly elevates the apparent value of creatine. The Folin method for creatinine-creatine, used with the precipitation method for creatinine, gives satisfactory results in all cases tried where irregularities occur in results by the Folin method. (b) The Myers and Benedict methods for creatine give high results, presumably due to the action of decomposition products, especially those arising from glucose.

3. The creatine hitherto reported in the urine of children, post partum women, diabetics, and those undergoing starvation, is shown in each case to be actually converted into creatinine, and as such to give the color reaction. The identity of the compound is thereby established.

To members of the Barnes Hospital staff in the Departments of Medicine, Pediatrics, Obstetrics, and Surgery, I wish to express my thanks for so kindly supplying the urine of the several cases included in this paper.

STUDIES OF AUTOLYSIS.

I. THE ACCELERATING EFFECT OF MANGANOUS CHLORIDE ON LIVER AUTOLYSIS.

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Since 1900, when Jacoby[1] initiated the intensive study of autolysis, a mass of data has accumulated relating to the effect of various substances upon the autolytic reaction of tissues.

Jacoby himself showed that the liver of a phosphorus-poisoned animal digests itself faster and more completely than that from the normal animal. Hedin and Rowland[2] in 1901 showed that the addition of acid to liver, spleen, lymph glands, kidney, and heart muscle accelerated the reaction and led to more complete digestion of the proteins than occurred in unacidified tissues. Ascoli and Izar[3] in 1908 showed that colloidal metals, Fe $(OH)_3$ and As_2S_3, produce the same effect. In 1909 Preti[4] found that salts of lead in certain concentrations increase liver autolysis. Later[5] he showed acceleration in the presence of salts of Fe, Mn, Au, Pt, Al, and Co; while salts of Cu, Ni, Cd, Mg, and Zn inhibit the reaction, and salts of Ca, Na, and K have no effect. In 1911 Schapiro[6] confirmed Preti's work with salts of Fe, connecting the liver necroses of pernicious anemia with the increased Fe deposited there as hemosyderin. Pollini[7] also repeated Preti's observation on the effect of Fe compounds and confirmed his results. Kepinow[8] in 1911 showed that iodine solutions increased liver autolysis whether injected first into the living animal or added to a normal liver hash. Kaschiwabara[9] in 1912, however, showed that the *in vitro* acceleration found by Kepinow was due entirely to the faulty technique em-

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[1] M. Jacoby: *Ztschr. f. physiol. Chem.*, xxx, p. 174, 1900.

[2] S. G. Hedin and S. Rowland: *ibid.*, xxxii, pp. 341 and 531, 1901.

[3] M. Ascoli and G. Izar: *Biochem. Ztschr.*, vii, p. 142, 1907; x, p. 356, 1908.

[4] L. Preti: *Ztschr. f. physiol. Chem.*, lviii, p. 539, 1909.

[5] Preti: *ibid.*, lx, p. 317, 1909.

[6] L. Schapiro: *Ztschr. f. exper. Path. u. Therap.*, xi, p. 355, 1912.

[7] L. Pollini: *Biochem. Ztschr.*, xlvii, p. 396, 1912.

[8] L. Kepinow: *ibid.*, xxxvii, p. 238, 1911.

[9] M. Kaschiwabara: *Ztschr. f. physiol. Chem.*, lxxxii, p. 425, 1912.

ployed. He could demonstrate no marked effect of iodine on the autolysis of a normal liver. He confirmed the effect produced by injecting iodine into an animal and later observing the rate of autolysis of the liver upon removal.

These typical examples serve to show what a wide range of substances are reported as accelerators of autolysis. There is little or no evidence of what the mechanism of this increased digestion may be. Much of the data, indeed, appears unreliable on account of the technique adopted. The method of heat coagulation and estimation of non-coagulable nitrogen in the filtrate is one most commonly found in the literature. Its unreliability under the varying conditions of the experiments is obvious. Where the technique was adequate the interpretations are frequently confused or not justified by the data. It seemed, therefore, desirable to repeat in some detail a few of the more suggestive experiments in the hope of arriving at some basis of interpretation of the results already obtained.

Perhaps the most common oversight to be found in the literature of autolysis is the failure to differentiate between acceleration of the reaction and change of equilibrium. A catalytic reaction may be enormously accelerated without involving any alteration of the point of final equilibrium, and a typical example of this is acceleration produced by an increase of the catalyst present. The inversion of cane-sugar may be greatly accelerated by increasing the concentration of acid or invertase present without appreciably altering the level at which the reaction comes to equilibrium. The presence of more catalyst or enzyme should not lead to any marked shift in the equilibrium of the reaction, except when a more or less stable combination is formed between the enzyme and substratum or between enzyme and products. In the autolytic reactions we may assume that the enzymes act approximately like true catalytic agents—at least until evidence to the contrary is obtained. In the presence of a large excess of water the reaction—PROTEIN+WATER⟵⟶PRODUCTS—should go to the complete hydrolysis of the protein. Where trypsin or erepsin is the enzyme, it is well known that we may get complete loss of the biuret reaction from a protein digest, made sufficiently dilute. Hydrolysis of the protein therefore approximates 100 per cent in such cases. With other

proteases under similar conditions of dilution we get approximately 100 per cent hydrolysis of the specific protein substratum. Tissue autolysis appears, on the other hand, to be an exception to this general rule. The exception, however, is more apparent than real, as we expect to prove.

The interpretation of his results given by Preti is that the metallic salts which he used with liver pulp were themselves catalytic agents. His figures, on the other hand, clearly show that the salts not only accelerate the reaction, but lead to a shift in the point of equilibrium toward more complete hydrolysis. The salts, therefore, cannot be catalytic agents. Hedin and Rowland, studying the effect of acids upon autolysis, suggest that either a zymogen is activated by the acid, or the enzyme already free is rendered more active. But activation of a zymogen should cause acceleration of the reaction without such pronounced shifts of equilibrium as they record. Increasing activity of an already active enzyme would be indistinguishable from an increase in amount of enzyme unless the specific nature of the enzyme itself were also changed,—and of this there is no positive evidence.

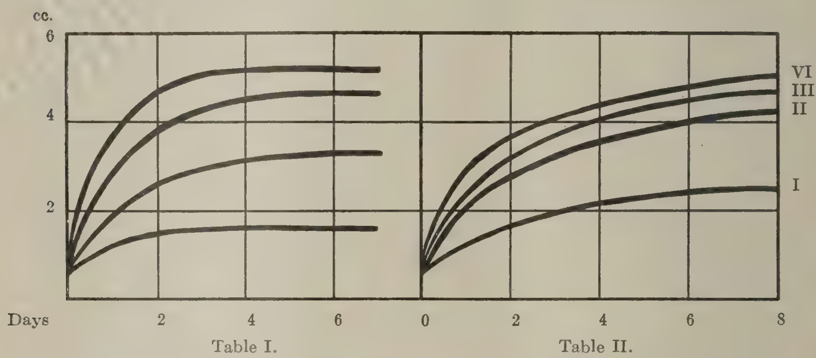
EXPERIMENTAL PART.

In the following experiments we have verified in detail one of these striking reaction changes induced by manganous chloride, and have attempted an explanation of it in terms of catalysis such that it can be broadly applied to many other similar phenomena. We believe that the experiment leads to a conception of the autolytic reaction which has very definite bearings upon its relation to atrophy, involution, and necrosis.

Fresh liver of dog or pig was ground to a smooth pulp in a hash machine. In some cases the pulp was rubbed through a twenty-mesh sieve or squeezed through a perforated die to remove shreds of connective tissue; in others the whole pulp was used. No essential difference was found between the sieved and the unsieved tissue; since the connective tissue is never digested, and merely increases the total protein nitrogen of the mixture. 50 gm. portions of this pulp were made up with toluene-water to 250 cc. With a blunt ended pipette, used throughout the series, a 25 cc. initial sample was taken at once, diluted with 25 cc. of water and 50 cc. of a tannic acid-salt solution made up according to Plimmer's formula.[10] After standing

[10] R. H. A. Plimmer: *Practical Physiological Chemistry*, London, 1910, p. 256.

for twelve hours or more the precipitated proteins were filtered off and the nitrogen was determined by the Kjeldahl method in 25 cc. of the filtrate. Where not otherwise specified the tabulated figures indicate the amount of $\frac{N}{5}$ HCl required to neutralize the ammonia distilled from these aliquots. The increase of such soluble nitrogen forms a safe basis for judging the rate and extent of protein hydrolysis. Digestions were kept at 37° C., shaken frequently to insure saturation with toluene, and from time to time further aliquots were removed and assayed. Total nitrogen was determined in 10 cc. of the digestion mixture, the sample being usually taken after autolysis had gone on for several days, so that a representative portion could be obtained. From these data the percentage proteolysis of each aliquot was calculated.



CURVE 1.

Experiment I. The effect of manganous chloride on dog liver autolysis.

The experiment shows that increasing the amounts of $MnCl_2$ present leads to a roughly proportional acceleration and shifting upwards of the equilibrium. While in the presence of the higher concentrations of the salt, the reaction has not fully reached equilibrium at the end of the experiment, it is at least approximated. Even in two or three days it is quite possible to distinguish between true acceleration and an acceleration accompanied by a shift of equilibrium. The control is usually within 10 per cent of equilibrium at the end of two days. It should be pointed out that even in ten days the reaction may not be at an absolute point of balance. There is usually a very slow continuation of the reaction after that time—almost nil in the con-

trols, quite appreciable in those containing considerable amounts of the salt. For all purposes the reaction is at equilibrium before ten days. The slow but measurable change after that is probably due to another factor which will later be explained.

TABLE I.

	$MnCl_2 \cdot 4H_2O$ per 25 cc. hash	Mol. concentration	$\frac{N}{5}$ HCl used for 25 cc. aliquot of tannic acid filtrate				Total nitrogen digested				Total N
			Day								
			0	1	2	7	0	1	2	7	
	gm.		cc.	cc.	cc.	cc.	per cent	per cent	per cent	per cent	cc.
I	0	0	0.47	1.25	1.52	1.60	5.6	16.8	20.5	21.5	11.6
II	0.25	$\frac{1}{200}$	0.72	2.00	2.63	3.30	9.7	26.9	35.3	44.4	11.7
III	0.50	$\frac{1}{100}$	0.65	—	3.9	4.65	8.7	—	52.4	62.5	12.3
IV	2.50	$\frac{1}{20}$	0.8	3.7	4.8	5.15	10.7	49.7	64.5	69.2	—
										Average	11.9

TABLE II.

	Mol. concentration $MnCl_2 \cdot 4H_2O$	$\frac{N}{5}$ HCl titrated				Total nitrogen digested				Total N on 10 cc. aliquot as $\frac{N}{5}$ HCl
		Day								
		0	1	3	8	0	1	3	8	
		cc.	cc.	cc.	cc.	per cent	per cent	per cent	per cent	cc.
I	0	0.55	1.30	1.80	2.50	6.98	16.51	22.86	31.75	
II	$\frac{1}{100}$	0.70	2.15	2.60	4.20	"	27.30	33.02	53.34	12.60
III	$\frac{1}{80}$	0.40	2.40	3.70	4.60	"	30.48	46.35	58.41	
IV	$\frac{1}{60}$	0.50	2.45	3.80	4.90	"	31.11	48.25	62.21	
V	$\frac{1}{40}$	0.50	2.80	3.90	5.55	"	35.55	50.16	70.48	
VI	$\frac{1}{20}$	0.60	3.00	4.00	5.00	"	38.47	51.43	63.48	12.60
	Average	0.54							Average	12.60

Experiment II. Determination of the optimum concentration of manganous chloride.

In both livers the optimum digestion required 1/10 mol. $MnCl_2$ present. Nos. I, V, and VI, dog liver, were allowed to continue autolyzing for a month. At the end of that time, the control,

TABLE III.

Dog liver.

	Mol. concentration $MnCl_2 \cdot 4H_2O$	$MnCl_2 \cdot 4H_2O$	$\frac{N}{5}$ HCl titrated				Total nitrogen digested				Total N in 10 cc. aliquot as $\frac{N}{5}$ HCl
			Day								
			0	1	4	10	0	1	4	10	
		per cent	*cc.*	*cc.*	*cc.*	*cc.*	*per cent*	*per cent*	*per cent*	*per cent*	*cc.*
I	0	0	0.70	1.55	1.95	2.32	7.60	17.20	21.64	25.74	14.55
II	$\frac{1}{50}$	0.4	0.65	2.80	4.90	6.20	"	31.07	54.37	68.79	14.55
III	$\frac{1}{25}$	0.8	0.60	4.00	5.70	6.22	"	44.39	63.25	69.01	
IV	$\frac{1}{15}$	1.48	—	4.00	5.40	6.57	"	44.39	59.92	72.90	
V	$\frac{1}{10}$	2.0	0.80	4.42	5.40	6.60	"	49.04	59.92	73.23	
VI	$\frac{1}{5}$	4.0	0.70	4.00	5.40	6.15	"	44.39	59.92	68.24	14.50
VII	$\frac{1}{2.5}$	7.92	0.70	3.80	5.20	5.80	"	42.16	57.70	64.35	14.10
	Average		0.69						Average		14.42

TABLE IV.

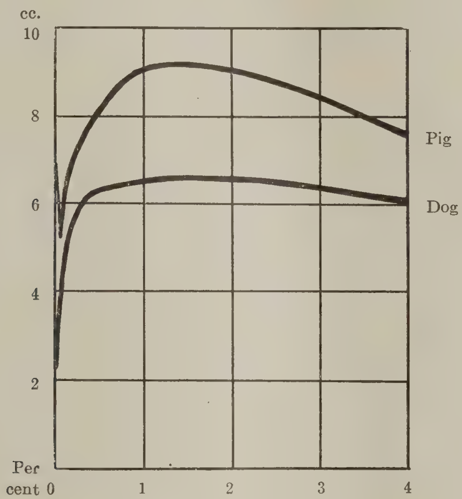
Pig liver.

	$ZnSO_4 \cdot 7H_2O$	$\frac{N}{5}$ HCl			$MnCl_2 \cdot 4H_2O$	$\frac{N}{5}$ HCl	
		Day				Day	
		0	10			0	10
	per cent	*cc.*	*cc.*		*per cent*	*cc.*	*cc.*
I	0	1.3	5.9	I	0	1.7	6.9
II	0.014	"	5.3	II	0.01	"	5.6
III	0.028	"	5.2	III	0.02	"	5.2
IV	0.057	"	5.0	IV	0.04	"	5.2
V	0.11	"	5.7	V	0.08	"	6.8
VI	0.28	"	5.8	VI	0.20	"	7.0
VII	0.57	"	6.5	VII	0.40	"	7.7
VIII	1.19	"	7.1	VIII	0.80	"	9.0
IX	2.87	"	6.5	IX	2.00	"	9.1
X	5.74	"	6.5	X	4.00	"	7.7
XI	11.9	"	5.7	XI	40.00	"	2.6
XII	28.7	"	5.3	XII	80.00	"	1.3

No. I, had increased from 25 to 27 per cent digestion; V had attained 87 per cent; and VI, 79 per cent. No. I still retained the pink color of unaltered hemoglobin with no apparent diminu-

tion in the mass of suspended, undissolved protein. In the other two nearly everything but shreds of connective tissue seemed to be in solution.

It will be seen that the zinc salt used has much the same type of effect on autolysis of pig liver as the salt of manganese. Its accelerating action is less pronounced and appears in a narrower range of concentration. It is interesting to note too that pig liver normally autolyzes much more completely than dog liver, and does not seem to be accelerated by low concentrations of



CURVE 2.

the manganese salt, but somewhat inhibited. Curve 2 shows the relation between the concentration of $MnCl_2$ and the approximate equilibria attained in ten day digestions.

Experiment III. Determination of the effect of manganous chloride on various livers.

The effect of $MnCl_2$ is evidently alike qualitatively on all mammalian livers tried. On the other hand, teleost livers, as exemplified by the dog-fish and sand-shark, are conspicuous by the very slight or negative effect produced. This bears out the

well known differences between teleost and mammalian liver. While the shark liver has a true hepatic function in that it secretes bile, it does not, on the other hand, store glycogen; it frequently contains enormous amounts of fat,—60 per cent of the moist weight,—and its enzymic content is very different from the liver of mammals.[11]

Acceleration of liver autolysis by manganese salts may be due to the following reactions,—assuming that the proteases act like true catalytic agents or as nearly so as does trypsin, for example.

I. The salt may activate a zymogen. This should require only very minute amounts of the salt, since the actual mass of enzyme

TABLE V.

SPECIES	MOL. CONCENTRATION $MnCl_2$	$\frac{N}{5}$ HCl Day 0	$\frac{N}{5}$ HCl Day 10	TOTAL	SPECIES	MOL. CONCENTRATION $MnCl_2$	$\frac{N}{5}$ HCl Day 0	$\frac{N}{5}$ HCl Day 10	TOTAL
				per cent					*per cent*
Dog 1	0	0.6	2.3	25.7	Pig 1	0	1.7	5.9	50.5
"	$\frac{1}{10}$	0.6	6.6	73.2	"	$\frac{1}{10}$	1.7	9.9	84.8
Dog 2	0	0.5	2.5	31.7	Pig 2	0	1.5	4.4	36.6
"	$\frac{1}{20}$	0.5	5.0	63.4	"	$\frac{1}{10}$	1.5	9.4	78.3
Rabbit	0	1.7	3.4	36.4	Dog-fish	0	4.8	7.2	62.6
"	$\frac{1}{10}$	1.7	5.7	61.1	"	$\frac{1}{10}$	4.8	7.2	62.6
Shark 1	0	2.9	3.5	49.4	Shark 2	0	4.0	5.3	53.0
"	$\frac{1}{10}$	2.9	3.9	55.1	"	$\frac{1}{10}$	4.0	6.7	67.0

must be very small indeed. Such an activation should cause acceleration without such pronounced changes in equilibrium. If the enzyme were a true catalyst in the theoretical sense, it should have no effect on equilibrium.

II. The salt may render the enzyme more active in the sense of being less specific, so that it can catalyze the hydrolysis of more of the proteins present. This should lead to acceleration and a shift upward of equilibrium.

III. The salt may provide a medium in the nature of a coferment, similar to hydrochloric acid and pepsin, in which an other-

[11] H. C. Bradley: this *Journal*, xiii, p. 407, 1912–13.

wise inactive enzyme or zymogen becomes active. If this new enzyme were also less specific than the normally active autolytic enzymes, it should result in more rapid and complete digestion.

IV. The salt may combine with the proteins present rendering them more labile or available as substratum for the existing enzymes. This would cause acceleration and shift of the final equilibrium toward greater hydrolysis. The optimum conditions should be reached after the addition of considerable amounts of $MnCl_2$; since the mass of protein present is large. Also since the salt evidently has little deleterious effect on the enzyme, even in relatively high concentrations, the continued addition of it should lead to gradual inhibition due to increased viscocity, changes in surface tension, etc.

Of these four mechanisms, No. I seems least likely. Small amounts of the salt appear to inhibit the digestion in some cases; in all the optimum concentration is high. The shifts upward in the equilibrium, amounting as they do to over 300 per cent, are too large to be attributed to enzyme-substrate or enzyme-products combinations. While such shifts are well known in enzyme reactions, as a rule they are of smaller magnitude.[12,13] Of the other hypotheses, the last appears to us to accord best with the facts observed. The large amount of the salt required for optimum results tends to negate II and III. The fact that the connective tissue remains undigested in all experiments suggests that the enzyme itself has not lost its erepsin-like specificity. This is further confirmed by the fact that ovalbumin or edestin added to these hydrolyses do not digest. If the enzyme had become a less specific protease we should expect these native proteins and the connective tissue of the gland to undergo hydrolysis.

In concrete form, our hypothesis follows. Liver pulp contains a variety of protein fractions: A. Connective tissue; collagen, reticulin, etc. This fraction is wholly insoluble under ordinary conditions, even in the presence of $MnCl_2$. It remains intact at the end of the longest autolysis. Though protein, it obviously cannot be considered as substratum. Its mass there-

[12] Bradley: *ibid.*, viii, p. 251, 1910.

[13] In the case of lipase we may get shifts of equilibrium with increased enzyme concentration which do attain large proportions.

fore has no effect on the reaction, though it will be represented in the total nitrogen of the digest. B. A protein fraction consisting probably of albumins, hemoglobin, nucleoproteins, and a mass of insoluble proteins, which are not normally hydrolyzed by the autolytic enzymes. Liver protease was shown by Jacoby[14] to be quite specific; by Vernon[15] to resemble erepsin. The B fraction is ordinarily unavailable for the specific protease of the liver and hence cannot be considered as substratum. Its presence in the liver autolysis can have no mass effect in determining speed of reaction or point of equilibrium. Like the connective tissue it has no influence on the autolytic reaction. C. Probably a globulin-like fraction, catalyzed by the normal enzymes of the tissue. (According to Jacoby, the liver globulin disappears during the course of autolysis, the albumin does not.) This fraction constitutes the real substratum; its mass determines the amount of non-precipitable nitrogen produced during autolysis since it is hydrolyzed completely. The liver of the pig contains a larger proportion of the C fraction than does that of the dog. Any addition to the C fraction—from without, or from within by the conversion of some of the B fraction into available protein—will result in a more rapid liberation of non-precipitable nitrogen, and a proportionately larger amount at the end of the reaction. The effect of the $MnCl_2$ in this series of experiments is, we believe, so to alter proteins of the B fraction that they become available in the reaction. The more of the salt added, the greater the mass of substratum thus added to the original C fraction until all of the protein susceptible to this change has been converted into digestible material. This is the optimum concentration of the salt, and further additions produce no effect. It is quite possible that during life the amount of the C fraction may be increased in the liver. Such a liver on removal will autolyze faster and farther than the normal. The effect of iodine injections, of lead or phosphorus poisoning, is probably so to alter the liver proteins that more become available to the autolytic enzymes.

From the values tabulated we see that the available C fraction

[14] Jacoby: *Ergebn. d. Physiol.*, i, p. 213, 1902.
[15] H. M. Vernon: *ibid.*, ix, p. 138, 1910.

amounts in the dog liver to 25 to 30 per cent of the total nitrogen. In pig liver it is frequently more. This fraction is indicated by the amount of hydrolysis in the normal controls. The C and B fractions together amount to 75 to 90 per cent of the total nitrogen, since that is the amount of hydrolysis after adding the optimum amount of $MnCl_2$. Proteins of the B fraction in the dog thus amount to about 50 to 60 per cent of the total nitrogen. Connective tissue constitutes about 10 to 25 per cent of the total nitrogen.

A normal liver hash after months of autolysis still remains thick and turbid with suspended insoluble proteins. Its color is the pink of hemoglobin, evidently untouched by the proteolytic enzymes. In the $MnCl_2$ treated samples the color disappears in one to two days, the turbidity clears up quickly, and at the end of a few weeks the digest separates into a clear pale yellow solution with the undigested connective tissue shreds collecting with the toluene at the top. It is certain that the great mass of protein material, left undigested in the control after months of standing in the incubator, is in such treated samples completely dissolved. The control autolysis does not stop because the enzyme is destroyed; it stops because there is no more substratum for the enzyme. That this is not a case of pseudo-equilibria due to inactivation of the enzyme before true equilibria are reached is shown in the following experiment.

Experiment IV.

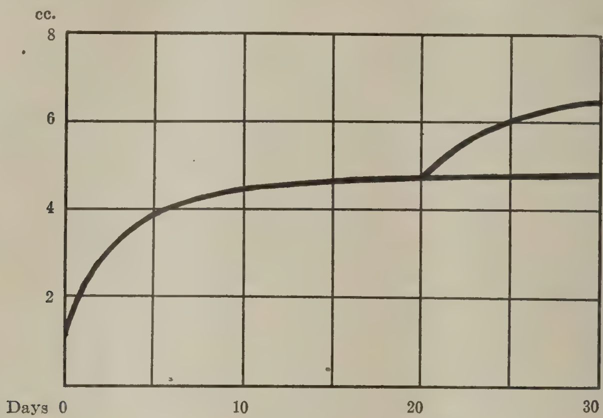
A liver hash was allowed to autolyze for twenty days. At the end of this period, when equilibrium had long since been attained or at least approximated, the hash was divided. One part was allowed to continue normally, another was treated with $MnCl_2$. Digestion was resumed in the latter.

TABLE VI.

	$\frac{N}{5}$ HCl titrated, Day 0	$\frac{N}{5}$ HCl titrated, Day 20	Mol. concentration $MnCl_2 \cdot 4H_2O$ added	$\frac{N}{5}$ HCl titrated, Day 24	$\frac{N}{5}$ HCl titrated, Day 30	Total N in 10 cc. aliquot as $\frac{N}{5}$ HCl
						cc.
I.	1.0	4.7	0	4.7	4.8	15.0
II.	1.0	4.7	$\frac{1}{10}$	5.9	6.5	15.0

The enzymes were therefore still active even after three weeks' autolysis.

The very slow continuation of the reaction after ten days is probably due to the gradual change of some of the insoluble B fraction into substratum. This may be brought about by slow hydrolysis due to water alone, or it may be due to an enzyme present in exceedingly minute amounts.



CURVE 3.

SUMMARY.

1. In the presence of a large excess of water, pig and dog livers autolyze until 25 per cent or more of the total nitrogen is non-precipitable by tannic acid. About 75 per cent remains indefinitely undigested, though the enzyme is still active for a long time after the reaction comes to approximate equilibrium.

2. If $MnCl_2$ is added to a liver digest, the reaction is so altered that from 75 to 90 per cent of the nitrogen is soluble in tannic acid. A residue of connective tissue chiefly—amounting to about 10 per cent of the total nitrogen—remains indefinitely undigested.

3. The addition of small and increasing amounts of the salt leads to proportionate shifts upward of the equilibrium until a concentration of 1/10 mol. is reached. Beyond this, increasing concentration of the salt gradually diminishes the speed and lowers the equilibrium point.

4. The effect of the $MnCl_2$ is believed to be due to its so altering the normally resistant fraction of liver proteins as to render them digestible by the protease present. The effective mass of substratum in the digest is thus increased by the presence of $MnCl_2$.

5. The pseudo-equilibria represent therefore true points of equilibrium, determined by the mass of proteins constituting the effective substratum present in each case. The other proteins present are unavailable and exercise no influence on the equilibrium of the reaction.

ON THE INFLUENCE OF BALANCED AND NON-BALANCED SALT SOLUTIONS UPON THE OSMOTIC PRESSURE OF THE BODY LIQUIDS OF FUNDULUS.

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I.

In former publications Loeb has described a method which offered an indirect proof for his assumption that a pure NaCl solution of a sufficiently high concentration rendered the cell membranes permeable, while the addition of $CaCl_2$ counteracted this destructive effect of a pure solution of NaCl. This method consisted in measuring the time during which the fertilized eggs of *Fundulus* were able to float on solutions of a higher specific gravity than the egg. Thus it was found that the egg will float on a mixture of 100 cc. 3 M NaCl + 1 cc. 10/8 M $CaCl_2$ for several days, during which time the embryo will continue to live and develop; while in a pure 3 M solution of NaCl the embryo will die and the egg will sink to the bottom of the test-tube in a few hours; and in a 10/8 M solution of $CaCl_2$ it will sink in less than one-half hour.[1] Loeb assumes that in the mixture of NaCl + $CaCl_2$ the embryo can live and the egg can continue to float, since the membrane remains intact for several days; for it can be shown that a mixture of 3 M NaCl + 1 cc. $CaCl_2$ kills the embryo of the young fish almost instantly after hatching. The embryo can, therefore, remain alive in such a solution because the membrane remains impermeable for the solution, while each of the constituents alone at this high concentration rapidly renders the membrane permeable and thus reaches the embryo. By the same method it was ascertained

[1] J. Loeb: *Science*, xxxvi, p. 637, 1912; *Biochem. Ztschr.*, xlvii, p. 127, 1912.

that after a brief treatment of the egg with an injurious solution it sank very rapidly when put into a mixture of 100 cc. 3 M NaCl + 1 cc. 10/8 M $CaCl_2$.

This method could not be applied to the fish itself and it seemed advisable to ascertain for this object by a direct test whether or not a pure NaCl solution of a sufficient concentration increases the permeability of the fish rapidly, while the addition of a trace of $CaCl_2$ counteracts this effect. This question could be decided by measuring the concentration of the body juice of the fish in isosmotic concentrations of balanced (*e.g.*, sea water or NaCl + $CaCl_2$) and non-balanced solutions (*e.g.*, NaCl).

Fundulus is too small to permit extensive work with the determination of the alteration of the freezing point of the blood. It was necessary to determine the alterations of the freezing point of the body juice of the fish, obtained with the aid of a Buchner press. The general method consisted in this: The fish were kept in a solution of a certain concentration and constitution for some time, and then, after having been washed several times for about five minutes in a porcelain filter with fresh water (to get rid of the solutions adhering to the surface of the fish and its gills), they were decapitated, cut into smaller pieces, and submitted to the Buchner press. The freezing point of the liquid obtained was determined.

II.

The fish were kept in artificially prepared sea water consisting of 100 molecules of NaCl, 2.2 molecules of KCl, 1.5 molecules of $CaCl_2$, 3.8 molecules of $MgSO_4$, and 7.8 molecules of $MgCl_2$. Since the sea water had to be prepared in large quantities no chemically pure salts were used. The freezing point depressions of this sea water for different concentrations were as follows:

	°*C.*
M/2 sea water	Δ = 2.071
M/4 sea water	Δ = 1.038
M/8 sea water	Δ = 0.540
M/16 sea water	Δ = 0.285

The fish were kept in M/2 sea water unless the contrary is stated. The pure NaCl solutions used had the following freezing point depressions:

	°C.
M/2 NaCl	$\Delta = 1.780$
M/4 NaCl	$\Delta = 0.895$

We took it for granted that the freezing point depression of the body juice of *Fundulus* kept in M/2 sea water would be a constant, but were surprised to find that this is not the case. It was generally noticed that the freezing point of the body juice of the fish became lower the longer they were kept in the laboratory. Thus in one case the body juice of the fish, immediately after they were brought into the laboratory and before they were put into the tanks, had a depression of $\Delta = 0.945°$. The fish were then distributed into three solutions of artificial sea water of different concentrations, M/2, M/8, and M/16. After three intervals of 1, 8, and 21 days the freezing point of their body juice in each of these solutions was determined.

TABLE I.

	Depression of freezing point of body juice of fish after		
	1 day	8 days	21 days
	°C.	°C.	°C.
In M/2 sea water	1.050	0.925	0.830
In M/8 sea water	0.900	0.935	0.825
In M/16 sea water	0.900	0.840	

In M/2 sea water the concentration of the body juice of the fish was diminished more than 25 per cent in three weeks. In M/8 sea water at first a slight rise but then also a diminution took place. In a second experiment of this kind the body juice of the fish when brought into the laboratory, before they were put into the M/2 sea water, had a freezing point depression of $\Delta = 0.875°$. They were then put into M/2 and M/8 sea water, respectively.

TABLE II.

	Depression of freezing point of body juice of fish after	
	3 days	16 days
	°C.	°C.
In M/2 sea water	0.925	0.800
In M/8 sea water	0.895	0.785

Again we notice that the freezing point depression becomes less the longer the fish remain in the laboratory. The difference in M/2 and M/8 sea water was small, as was to be expected since *Fundulus* lives equally well in M/2 and M/8 sea water. The body juices of the fish become less concentrated the longer the fish lives in the aquarium, and this diminution therefore is in wide limits independent of the concentration of the outside solution. We thought at first that we were the victims of definite errors due to differences in the way of washing the fish, but control experiments eliminated this explanation. It finally occurred to us that the lack of food might be the cause of this change, since the fish were not fed in the laboratory. A test seemed to confirm this suspicion. A set of fish brought into the laboratory on November 23 were kept under the same conditions and without food, each in M/2 sea water. After three weeks the freezing point depression of their body juice was found to be 0.795°. They were then divided into two lots; one was fed regularly with fish food, the other remained unfed. The result is contained in Table III.

TABLE III.

FREEZING POINT DEPRESSION OF FISH IN M/2 SEA WATER AFTER	FED	UNFED
	°C.	°C.
2 days	0.820	0.795
8 days	0.890	0.740

After eight days of feeding the freezing point depression had increased considerably, while in those not fed the freezing point had diminished further.

It is not possible to express any suggestion as to the source of the effect which starving has on the freezing point depression, though it seems more promising to look for the cause of these variations in the organic rather than the inorganic soluble constituents of the fish. As a result of our observations we may state that the osmotic pressure of the body juice of *Fundulus* in M/2 or M/8 sea water is not a constant, but diminishes apparently with continued starvation. It varies roughly between $\Delta = 1.00°$ and $\Delta = 0.78 - 0.74°$ or a little less. Roughly speaking, it is a little below that of M/4 sea water and may go 25 per cent below this limit.

The results show that in experiments of this kind the freezing

point of the body liquid in normal sea water has to be determined as a control in each case, especially if the fish are not fed as in our experiments.

III.

The influence of varying concentrations of the sea water upon the osmotic pressure of the body juices of Fundulus.

Loeb has shown that this fish can live equally well in solutions from 10/8 M to M/128 sea water.[2] It is necessary to bring the fish not too suddenly from normal into the 10/8 M sea water. It was of interest to find out the possible changes which occur in the concentration of the body juices of these fish in sea water of different concentrations. Fish brought in at the same time were distributed into various dilutions of sea water. Table IV gives the result in dilute solutions.

Fundulus were kept for eight days in sea water of the following concentrations: M/2, M/4, M/16, M/32, M/64, and M/128. The osmotic pressure of the body juice was determined after one day, and after eight days. (The fish had been in the laboratory for eighteen days previous to the beginning of the experiment.)

TABLE IV.

	Δ AFTER 1 DAY	Δ AFTER 8 DAYS
	°C.	°C.
M/2 sea water	0.850	0.790
M/4 sea water	0.810	0.820
M/8 sea water	0.780	0.760
M/16 sea water	0.755	0.735
M/32 sea water	0.770	0.770
M/64 sea water	0.765	0.730
M/128 sea water	0.715	0.750

The influence of the dilution of the sea water is more marked after one day than after eight days. If we make allowance for irregularities due to unknown internal causes we notice that there is after eight days only a slight if any difference between the body liquids of the fish kept in sea water varying between M/2 and M/128, and this agrees with the fact that in all these solutions the fish can live indefinitely with the possible exception of the weakest solution.

[2] Loeb: *Biochem. Ztschr.*, liii, p. 391, 1913.

The statement occasionally made that the blood of euryhaline[3] fish has a lower osmotic pressure when they are in dilute sea water is not correct for the body liquid of *Fundulus*, when this teleost has been kept for some time in the medium; while a slight influence of the concentration may show itself earlier.

With the same lot of fish some experiments had previously been made with sea water of a higher concentration; namely, 6/8 M and 10/8 M, after one day and later. All the fish had first been in M/2 sea water and the concentration of the sea water was gradually raised (each day by almost but not quite M/8) until it had the desired concentration.

TABLE V.

	Δ AFTER 1 DAY	Δ AFTER 6–8 DAYS	Δ AFTER 9 DAYS
	°C.	°C.	°C.
M/2 sea water	0.925	0.850	
6/8 M sea water	1.025	0.880	
8/8 M sea water	1.190	0.945	
M/2 sea water	0.850	0.800	0.825
10/8 M sea water	1.190	1.195	1.040

In another experiment after one day Δ was in 6/8 M sea water 0.915; after five days 0.855.

For the sake of completeness we wish to add a series of measurements made two years ago.

TABLE VI.

Lowering of the freezing point of the body juice of Fundulus.

	Δ °C.
In M/80 NaCl + KCl + $CaCl_2$ after 4 days	1.01
In M/80 NaCl + KCl + $CaCl_2$ after 1 day	0.97
In M/80 NaCl + KCl + $CaCl_2$ after 1 day	0.99
In M/8 NaCl + KCl + $CaCl_2$ after 7 days	0.95
In M/2 NaCl + KCl + $CaCl_2$ after 7 days	0.98
In M/2 sea water after a number of days	1.00
In M/2 sea water after a number of days	1.00
In M/2 sea water after a number of days	0.96
In M/2 sea water after a number of days	1.01
In 10/8 M NaCl + KCl + $CaCl_2$ adapted gradually	1.12

[3] This term denotes the fact that the fish can live in concentrations varying within wide limits.

The results agree with those given before, except that Δ was in general slightly higher, possibly because the fish had been fed regularly, though we have failed to keep a record on this point.

The result is very characteristic. During the first day the body juices of the fish change in concentration in the same sense with the change in the concentration of the medium. After some time this difference disappears or is greatly diminished. This diminution is less noticeable in the 10/8 M sea water than in the M/1 or 6/8 M sea water. This may possibly be explained by the fact that the fish were kept at a comparatively high temperature and hence were not quite normal in 10/8 M sea water. This point will be further investigated. It is, therefore, possible that the change in the concentration of the sea water increases transitorily the permeability of the skin, but that this transitory increase in permeability is rapidly repaired and leads to no injury of the fish.

IV.

We now come to the main problem of this paper; namely, whether the permeability is increased more if we put the fish into a non-balanced solution than if we put it into a balanced solution of the same concentration. For the sake of briefness we consider a solution of one sodium salt, *e.g.*, NaCl or NaBr or Na_2SO_4, as a non-balanced solution, while the addition of a trace of $CaCl_2$ makes the solution more balanced. NaCl + KCl + $CaCl_2$ in the proportion in which these salts exist in the sea water is more balanced than NaCl + $CaCl_2$ without KCl, and sea water is a completely balanced solution. It should also be realized that as long as a pure NaCl solution has a very low concentration it is not very toxic, *i.e.*, the fish can live very long if not indefinitely in such a solution, and hence it is not possible to show that the addition of $CaCl_2$ improves the solution, though this may be the case. If, however, the concentration of NaCl increases, it becomes more toxic, and in this case the addition of $CaCl_2$ prolongs the life of the fish considerably. It should also be remembered that the toxic limit of the solution of different Na salts varies with the anion; thus Na_2SO_4 is more toxic than NaCl, and $NaNO_3$ more toxic than either; *i.e.*, it requires a lower concentration of Na_2SO_4, and a still lower concentration of $NaNO_3$, to kill the fish in a definite time.

The theory formerly expressed by Loeb consisted in this, that NaCl (or Na_2SO_4 or $NaNO_3$) should increase the permeability of the skin of the fish more rapidly when alone in solution than when $CaCl_2$ is added. When, therefore, the toxic solution is hypertonic (compared with the concentration of the liquids of the body) the concentration of the body juices of the fish should be increased more rapidly in a pure solution of NaCl than in a mixture of NaCl + $CaCl_2$ of the same osmotic pressure; and if the toxic concentration has a lower concentration than that of the liquids of the body the decrease in the concentration of the body juices of the fish should be more rapid in the pure solution of the toxic salt than if an antagonistic salt is added.

Moreover, it should be expected that the toxic solution gradually increases the permeability, and that the higher the concentration of a given toxic solution, the more rapidly should the permeability increase. The change in the permeability precedes the change in the concentration of the body liquid.

In a pure 6/8 M NaCl solution the fish will die in a few hours; in an 8/8 M solution they will die in less than one-half hour. In a M/2 NaCl solution they may live a day; sometimes a few may live two days or a little longer.

	°C.
Δ of fish 0.5 hour in M/1 NaCl (all dead)	1.490
Δ of fish 1.5 hours in 6/8 M NaCl (1 normal, 4 sick, 1 dead)	1.410
Control: Δ of fish in M/2 sea water	0.950

Another experiment of the same kind gave the following result:

	°C.
Δ of fish 0.5 hour in 8/8 M NaCl (4 normal, 4 lying on side)	1.255
Δ of fish 1.5 hours in 6/8 M NaCl (4 normal, 5 lying on side)	1.270
Control: Δ of fish in M/2 sea water	0.820

The rise of concentration of the body juice of the fish in a 6/8 M and 8/8 M NaCl solution is therefore enormous.

The experiment with a 6/8 M NaCl solution was repeated twice with the same result.

	°C.
Δ of fish 2 hours in 6/8 M NaCl (3 normal, 1 sick)	1.160
Δ of fish 3 hours in 6/8 M NaCl (3 normal, 1 sick)	1.135

In one experiment in M/2 NaCl the result was as follows:

	°C.
Δ of fish 24 hours in M/2 NaCl	1.095
Δ of fish 42 hours in M/2 NaCl	1.160
Δ of fish 24 hours in M/2 sea water	1.020

The concentration of the body juice of the fish therefore is higher in M/2 NaCl than in M/2 sea water, and it rises the more the longer the fish remains in the M/2 NaCl solution, indicating that the change of the concentration of the liquid of the body accompanies or follows the alteration of the surface of the body. *In all these experiments it was obvious that as soon as the fish began to lie on their sides the osmotic pressure of their body juices had increased.* The toxic effect of the solutions and the increase in permeability were closely associated.

The question may be asked whether in these experiments the increase in osmotic pressure noticed in such fish when kept in hypertonic NaCl solution was not the consequence of this condition of sickness. The same type of sickness can be produced quickly if we cause the fish to suffocate by replacing the air in the solution by hydrogen. Control experiments showed that fish rendered "sick" in a pure NaCl solution by driving out the air from the solution did not show any greater alteration of the freezing point of their body juices than fish in the same NaCl solution which had remained in contact with air and which were still normal. Hence it is obvious that the fact that the fish began to lie on their sides in the pure NaCl solutions was the consequence but not the cause of the increased permeability of their skin.

We now wish to compare with this the behavior of the fish in physiologically balanced solutions.

Experiment 1.

	°C.
Δ of fish 1.5 hours in 6/8 M NaCl (2 normal, 2 lying on side)	1.350
Δ of fish 1.5 hours in 100 cc. 6/8 M NaCl + 1 cc. 6/8 M $CaCl_2$ (all normal)	1.010
Δ of fish 1.5 hours in 6/8 M sea water	1.070
Δ of fish 1.5 hours in M/2 sea water	0.990

Experiment 2.

	°C.
Δ of fish 1.5 hours in 6/8 M NaCl (all but 1 normal)	1.110
Δ of fish 1.5 hours in 6/8 M sea water (all normal)	0.890
Δ of fish 1.5 hours in M/2 sea water	0.835

Experiment 3.

	°C.
Δ of fish 2 hours in 6/8 M NaCl (5 normal, 3 sick)................	1.320
Δ of fish 2 hours in 6/8 M sea water (all normal)..................	0.990

It is obvious that in hypertonic *physiologically balanced solutions the concentration of the body juice of Fundulus rises considerably less than in the non-balanced solutions of the same concentration.*

We will now take up experiments with weaker solutions in which the toxic effects appear much later.

Experiment 4.

	°C.
Δ of fish 4 hours in 5/8 M NaCl (all normal)......................	1.105
Δ of fish 4 hours in 100 cc. 5/8 M NaCl + 1 cc. 5/8 M $CaCl_2$ (all normal)	0.975

Experiment 5.

	°C.
Δ of fish 20 hours in M/2 NaCl....................................	1.050
Δ of fish 22 hours in 100 cc. M/2 NaCl + 1 cc. M/2 $CaCl_2$...........	0.950

In these and weaker solutions the difference in the action of the non-balanced and balanced solution became marked when the injurious effects of the non-balanced solution began to show themselves. It was therefore necessary to compare the effect of pure NaCl solutions of lower concentrations than M/2 for different periods of time.

Experiment 6.

	°C.
Δ of fish 17 hours in 3/8 M NaCl (all normal)......................	0.860
Δ of fish 18.5 hours in 100 cc. 3/8 M NaCl + 1 cc. 3/8 M $CaCl_2$ (all normal)..	0.850
Δ of fish 3 days in 3/8 M NaCl (1 died, rest normal; only the normal fish used for determination)....................................	1.085
Δ of fish 3 days in 100 cc. 3/8 M NaCl + 1 cc. 3/8 M $CaCl_2$ (all normal)	0.915

After three days, when the toxic effect of the pure NaCl solution began to show itself, the influence on the concentration of the body juice was also marked.

Experiment 7.

	°C.
Δ of fish 20 hours in M/4 NaCl (all normal)	0.880
Δ of fish 22 hours in 100 cc. M/4 NaCl + 1 cc. M/4 $CaCl_2$ (all normal)	0.875
Δ of fish in M/2 sea water	0.855
Δ of fish 4 days in M/4 NaCl (all normal) *a*	1.000
Δ of fish 4 days in M/4 NaCl (all normal) *b*	0.970
Δ of fish 4 days in 100 cc. M/4 NaCl + 1 cc. M/4 $CaCl_2$ (all normal)	0.860

In a M/4 solution of NaCl only after about four days will the injurious effects appear, and at this time we also notice that in the pure M/4 solution of NaCl the osmotic pressure of the body juice of the fish increases. Some of the NaCl apparently enters the fish.

In pure solutions of M/8 NaCl or below this concentration *Fundulus* lives for weeks or months (if not indefinitely), which means in the sense of Loeb's theory that in such a solution the permeability of the surface of the fish is not perceptibly altered for such a period of time; and hence we should expect that in these cases the addition of $CaCl_2$ to an NaCl solution does not alter the permeability. This was found to be the case.

Experiment 8.

	C.
Δ of fish 20 hours in M/8 NaCl (all normal)	0.815
Δ of fish 20 hours in 100 cc. M/8 NaCl + 1 cc. M/8 $CaCl_2$ (all normal)	0.795
Δ of fish 4 days in M/8 NaCl (all normal)	0 840
Δ of fish 4 days in 100 cc. M/8 NaCl + 1 cc. M/8 $CaCl_2$ (all normal)	0.775

V.

Experiments with Na_2SO_4 and NaBr.

The fish do not live long in a M/4 solution of Na_2SO_4, and as a rule they soon die even in a M/8 solution of Na_2SO_4; M/50 or M/20 solutions are tolerated for a longer time. The following table gives the results of some of the experiments.

TABLE VII.

△ OF BODY JUICE OF FUNDULUS AFTER	°C.
5 days in M/50 Na_2SO_4	0.815
6 days in M/50 sea water	0.815
48 hours in M/20 Na_2SO_4	0.765
50 hours in M/20 sea water	0.855
20 hours in M/10 Na_2SO_4	0.775
22 hours in M/10 sea water	0.875
2.5 hours in M/4 Na_2SO_4 (fish began to turn on side)	0.930
2.5 hours in M/4 sea water	0.820
1.5 hours in M/2 Na_2SO_4	1.125
1.5 hours in M/2 sea water	0.890

These experiments show a definite result. Whenever the osmotic pressure of the pure Na_2SO_4 solution is higher than the normal concentration of the body juice of the fish (M/2 and M/4 Na_2SO_4) it raises the osmotic pressure of the liquids of the fish more than is done by the corresponding concentration of sea water; whenever the concentration of the Na_2SO_4 solution is lower than the concentration of the body juices of the fish (M/10 Na_2SO_4 and less) the reverse is the case, although the difference is small. When finally the concentration of Na_2SO_4 becomes so low that it ceases to be toxic (M/50 Na_2SO_4) it behaves osmotically like sea water of the same concentration. This shows that a pure Na_2SO_4 solution of sufficiently high concentration makes the fish more permeable.

In a former paper Loeb has shown that the addition of $CaCl_2$ or $CaCl_2$ + KCl renders a pure Na_2SO_4 solution less harmful. Accordingly we should expect that in the latter case the permeability is altered less than in the former case.

TABLE VIII.

	△ OF BODY JUICE OF FUNDULUS AFTER	°C.
Hypertonic solutions	3 hours in M/4 Na_2SO_4 (fish normal)	0.910
	3 hours in 100 cc. M/4 Na_2SO_4 + 1.5 cc. M/2 $CaCl_2$ + 2.2 cc. M/2 KCl (normal)	0.880
	16.5 hours in M/4 Na_2SO_4 (fish lying on side but alive)	1.030
	18 hours in 100 cc. M/4 Na_2SO_4 + 1 cc. M/2 $CaCl_2$ (majority normal)	0.970

TABLE VIII—Continued.

	Δ OF BODY JUICE OF FUNDULUS AFTER	°C.
Hypotonic solutions	20 hours in M/8 Na_2SO_4 (mostly normal)	0.750
	20 hours in 100 cc. M/8 Na_2SO_4 + 1 cc. M/2 $CaCl_2$ (all normal)	0.810
	1 day in 2/25 M Na_2SO_4 (all normal)	0.720
	1 day in 100 cc. 2/25 M Na_2SO_4 + 0.75 cc. M/2 $CaCl_2$ (all normal)	0.860
	3 days in 2/25 M Na_2SO_4 (all normal)	0.800
	3 days in 100 cc. 2/25 M Na_2SO_4 + 0.75 cc. M/2 $CaCl_2$ (all normal)	0.840
	1 day in M/20 Na_2SO_4	0.770
	1 day in 100 cc. M/20 Na_2SO_4 + 0.75 cc. M/2 $CaCl_2$	0.825
	4 days in M/20 Na_2SO_4	0.765
	4 days in 100 cc. M/20 Na_2SO_4 + 0.75 cc. M/2 $CaCl_2$	0.820

These experiments prove that the addition of $CaCl_2$ diminishes or inhibits the increase of permeability induced by weak solutions of Na_2SO_4. The effect, however, is slight.

Very low concentrations of NaBr solutions cause the animals to lie on one side and ultimately cause their death, while the addition of $CaCl_2$ inhibits this effect. Since all these solutions are strongly hypotonic it was to be expected that if $CaCl_2$ is added to the solution the concentration of the body juices of the fish is higher than without the addition of $CaCl_2$. This is the case.

TABLE IX.

Δ OF BODY JUICE OF FUNDULUS AFTER	°C.
1 day in M/50 NaBr (all normal)	0.820
1 day in 100 cc. M/50 NaBr + 0.75 cc. M/2 $CaCl_2$ (all normal)	0.860
3 days in M/50 NaBr (all lying on side)	0.750
3 days in 100 cc. M/50 NaBr + 0.75 cc. M/2 $CaCl_2$ (all normal)	0.825
1 day in M/25 NaBr (all normal)	0.830
1 day in 100 cc. M/25 NaBr + 0.75 cc. M/2 $CaCl_2$ (all normal)	0.845
3 days in M/25 NaBr (all lying on side)	0.790
3 days in 100 cc. M/25 NaBr + 0.75 cc. M/2 $CaCl_2$ (all normal)	0.840

VI.

These experiments do not all give an equally positive answer to the question whether or not physiologically balanced solutions increase the permeability of the fish. The answer was most strikingly positive in the case of hypertonic NaCl solutions. The

answer seemed positive but was certainly less clearly so in the case of Na_2SO_4 or NaBr solutions (and we may also add in the case of $NaNO_3$, KCl, and of acid, though we are not giving the figures in this paper).

We can understand the fact if we bear in mind that although the non-balanced solutions alter the permeability, the death of the fish is not due to a change in the osmotic pressure of the body juices but to another effect of these substances, which requires only a slight alteration and slight increase of permeability of the epithelium of the gills, without altering the water contents of the fish perceptibly. The writers, in a former paper, examined the toxic effects of acid on fish and came to the conclusion that very weak acid solutions kill the fish by making the gills unfit for respiration (*e.g.*, by causing an excessive mucus secretion or by otherwise agglutinating the gill leaves).[4] This presupposes an alteration of the epithelium of the gills, but the fish die from suffocation long before any greater osmotic changes than those taking place in a neutral H_2O solution have had time to occur.

When the fish is put into a pure solution of $NaNO_3$, or NaBr, or KCl, etc., traces of these salts probably diffuse to the nerves or muscles of the gill or into the internal ear and possibly along the auditory nerve to the medulla. We may expect in this way a toxic action of the salt long before a great change in the osmotic pressure of the body juices has taken place. According to our figures, a slight increase in the permeability of the epithelium of the gills precedes this effect of Na_2SO_4, NaBr, etc., on the nerve elements which is prevented through the addition of $CaCl_2$. It is not excluded, however, that the antagonistic action of $CaCl_2$ in this case is not entirely due to a lowering of the permeability. Only in the case of the hypertonic solutions of NaCl do we notice great osmotic changes in the fish before it is killed. This is due in our opinion to the fact that the direct toxic effects of this salt on the nerves and muscles take place only when its concentration in the gills or in the brain cavity is comparatively high.

It is, therefore, obvious that death in all these cases is not due to the increase in the concentration of the body juice but to the toxic salts which diffuse into the gills (or the internal ear and

[4] J. Loeb and H. Wasteneys: *Biochem. Ztschr.*, xxxix, p. 167, 1912.

brain cavity?) and which kill the fish by stopping respiration through influencing the medulla or the nerves or muscles of the gills. But in all cases the diffusion of these salts to the nerves or muscles of the gill or the internal ear or the brain cavity presupposes an increase of the permeability of the gills, although this can not be proved in all cases.

Fundulus differs from most fish in that it is within wide margins independent of the osmotic pressure of the sea water; but it is rapidly killed when suddenly put from normal sea water into sea water of a concentration above 9/8 M. Our experiments seem to indicate that such solutions also increase the permeability of the epithelium of the gills or the whole fish. In other words, a balanced solution ceases to be balanced when its concentration exceeds a certain limit. This is sufficiently intelligible on the basis of Loeb's former experiments which showed that $CaCl_2$ can antagonize a solution of any other salt only as long as this solution does not exceed a certain concentration. For *Fundulus* this limit is high when NaCl is the toxic salt. For other fish the limit is much lower, and they can stand sea water, which is a physiologically balanced solution, only in very low concentrations. This is the case with the majority of fresh water fish, which die rapidly when put into ordinary (M/2) sea water. Paul Bert found that such fish die from suffocation and that their death is caused by "an exosmotic effect upon the gills whose epithelium becomes pale and where the circulation stops. The salt of the sea water withdraws water from the epithelium and the proper tissue of the gills and the finest ramifications of the blood vessels are obliterated, either by direct action upon the surrounding tissue and their contractile fibers or reflexly through the vasomotor nerves."[5] In addition thrombosis might occur. Backman has recently published some observations on the behavior of marine dogfish when put into hypotonic solutions. He found that if the sea water in which these fish live (with $\Delta = 1.880°$) is diluted to $\Delta = 0.5°$, after fifteen minutes the tension of O_2 in the blood is diminished from 19.1 to 3.7 per cent O_2. He assumes that "the hypotonic medium probably leads to a rapid

[5] P. Bert: *Compt. rend. Acad. d. sc.*, lxxiii, pp. 382 and 464, 1871; xcvii, p. 133, 1883.

injury of the gills whereby the absorption of O_2 is inhibited considerably."[6] For these fish sea water in a lower concentration probably alters the epithelium of the gills by causing cytolysis (?).

SUMMARY.

1. The experiments published in this paper show that the concentration of the body juice of *Fundulus* is influenced more strongly by non-balanced solutions than by balanced solutions of the same concentration. This supports the theory of Loeb that non-balanced solutions increase the permeability of the cells.

2. This influence of the non-balanced solutions on the concentration of the body juice of *Fundulus* is more obvious when the non-balanced solution is a pure (hypertonic) NaCl solution than when it is a solution of any other salt (Na_2SO_4, NaBr, etc.).

3. This finds its explanation in the fact that the fish do not die in a non-balanced solution from the change in the osmotic pressure of their body juice but from the action of the toxic salt on the epithelium, nerves, and muscles of the gills, or by action on the inner ear and medulla, which interferes with and finally stops the respiration. In the case of a very toxic salt the fish is killed before a marked change in the osmotic pressure of the body juice has taken place; while in the case of the less toxic NaCl great osmotic changes may take place in the fish before the salt will kill it.

4. Balanced solutions, *e.g.*, sea water, when applied in excessive concentrations, also increase the osmotic pressure of the body juice of the fish; possibly by first increasing the permeability of the gills (and the skin) of the fish.

5. When *Fundulus* are kept in M/2 sea water for some length of time the osmotic pressure of their body juice diminishes constantly to a certain limit, apparently as a consequence of starvation, since feeding seems to inhibit this decline.

[6] E. L. Backman: *Zentralbl. f. Physiol.*, xxviii, p. 495, 1914.

THE RELATION OF THE QUALITY OF PROTEINS TO MILK PRODUCTION.[1]

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Since the time of Wolff the feeding standard for milch cows has called for a narrow nutritive ratio; that is, a high percentage of digestible proteins in the ration. This standard has been made to vary with the production, ranging from 1:4.5 for a daily yield of 27.5 pounds of milk to 1:6.7 for 11 pounds of milk. These ratios refer to the proportion of digestible proteins to digestible carbohydrates and fats, the latter reduced to a carbohydrate basis. Experimental work[2] covering a period of nine years, as well as the experience of many dairymen, has even dictated the possibility of somewhat widening this ratio for economical production, and a ratio of 1:8.5 has sometimes been found economical where low producing animals are involved.

It is, of course, generally agreed that the "nutritive ratio" will vary with the individual's capacity to produce, this merely meaning that for a large production more proteins must be consumed than for a moderate or low production. In all the work that has been done on the protein requirements for milk production, no attention, as far as we are aware, has been given to the *quality* of the proteins used. Total digestible protein has been the guide.

With the development of protein chemistry by Kossel, Fischer, Abderhalden, and others came the present well known view of the

[1] Published by permission of the Director of the Agricultural Experiment Station.

[2] F. W. Woll and G. C. Humphrey: *Wisconsin Agricultural Experiment Station Research Bulletins*, No. 13, 1910.

amino-acid structure of these substances and their qualitative and quantitative differences in amino-acid content. Further developments in the nutritive relations of the proteins have shown that they differ in their value for maintenance and growth. The investigations of Osborne and Mendel[3] with purified proteins have added much to our knowledge of this phase of the subject, and the work in this laboratory of McCollum[4] on the naturally occurring proteins has emphasized the large quantitative difference in their efficiency for growth with swine. It was shown in this work that the proteins from milk when fed alone showed a retention of approximately 65 per cent of the nitrogen, while the nitrogen in the proteins from the cereal grains was retained to the extent of about 25 per cent. Supplementing the corn grain proteins with the proteins from the flaxseed (oil meal) greatly improved their efficiency. When the proteins of the ration consisted of 75 per cent from the corn kernel and 25 per cent from oil meal, the efficiency of the nitrogen for storage was increased to 40 per cent (unpublished data). In other experiments from this laboratory,[5] with calves, it has also been shown that the nitrogen of corn and alfalfa when used alone has an efficiency of about 25 per cent, while skim milk proteins show a retention of about 65 per cent of their nitrogen.

This work puts a new interpretation on the "nutritive ratio" of the animal feeder and the necessity for the accumulation of such data with our natural products. These data must not only be accumulated for growth, but the time has come for exact studies of the efficiency of the naturally occurring proteins from various sources for milk production. Whether the data accumulating on the efficiency of proteins for growth will agree or be in the same order as those secured for milk production is problematic. Special synthetic functions on the part of the mammary gland may come into such prominence as to make it less necessary that all building units for milk protein formation be supplied in the

[3] T. B. Osborne and L. B. Mendel: this *Journal*, xvii, p. 325, 1914; xviii, p. 1, 1914; xx, p. 351, 1915.

[4] E. V. McCollum: *ibid.*, xix, p. 323, 1914.

[5] E. B. Hart, G. C. Humphrey, and F. B. Morrison: *ibid.*, xiii, p. 133, 1912–13. H. Steenbock, V. E. Nelson, and E. B. Hart: *ibid.*, xix, p. 399, 1914.

ration. The mammary gland has specific synthetic functions now well recognized. The occurrence of lactose, special proteins such as casein and lactalbumin, and the lower fatty acids in milk are evidences of special constructive functions on the part of this gland, but in any case this may imply only an ability to construct from simple units already supplied in the blood stream rather than the construction of the unit itself. Osborne and Mendel[6] have, however, published one very interesting experiment where gliadin was the only protein in the diet of a pregnant and afterwards nursing rat. As is well known, this protein is practically free from the diamino-acid lysin, and upon this fact its inability to support growth probably rests. Yet in this record of a single rat, young were developed, milk was produced, and the young were apparently nourished adequately for thirty days. This must have called for special synthetic action on the part of the mammary gland unless the necessary complexes were drawn from the mother's tissues, which hardly seems possible considering the time that the young were suckled and the growth made by them. Not only was there evidence here of the synthesis of an amino-acid by the mammary gland, but also of purines and pyrimidines, constructed somewhere in the mother for the developing young.

While the above observation gives us some evidence of a certain degree of special synthetic action on the part of the mammary gland, there is no experimental evidence from any source of the extent of this special synthetic power for other amino-acids.

EXPERIMENTAL PART.

In the experiments to be described natural food products were used. While evidence collected in this manner would not give specific information as to just what chemical groups could or could not be synthesized by the mammary gland, it would, however, give evidence of whether there were differences in the efficiency of proteins from different sources for milk production. Not only would such information be of theoretical interest, but, in addition, of very great practical value. The purchase of protein concentrates by the American farmer is a large one, and

[6] Osborne and Mendel: *ibid.*, xii, p. 473, 1912.

information on the efficiency of proteins for milk production would be of special interest to him.

In our earlier work[7] it had been shown that there was practically no difference in the efficiency of the nitrogen of the corn grain and alfalfa hay for milk protein building. This work was particularly of interest for the information it gave concerning the value of the so called "amide" nitrogen of alfalfa hay. In the work to be reported here only the corn and wheat proteins have been compared with milk proteins. Preliminary to more extended investigations, it was believed desirable to work with these natural materials in order to learn whether there would be any difference in the efficiency for milk building of the proteins now known to differ very greatly in their efficiency for growth. If a difference did occur then the investigation of other materials would be warranted. This research indicates that there is a considerable difference in the efficiency for milk production of the proteins from the sources studied, and consequently other natural materials, especially the common concentrates, will be investigated as rapidly as opportunity affords.

Cows were used for the work. This necessitated the use of a roughage with a low content of digestible protein. The amount of roughage was kept constant and probably did not furnish more than 15 per cent of the nitrogen absorbed from the tract.

It was planned to use a rather wide nutritive ratio—about 1:8—as this would bring out more definitely any essential differences in the efficiency of the proteins as reflected in the amount of product produced. The selection of a 1:8 nutritive ratio made the digestible proteins about 7 per cent of the ration, and the total protein varied from 9 to 10 per cent.

Quantitative nitrogen balances were kept, as well as determinations of fat, solids, and the total proteins in the milk. Daily nitrogen determinations were made on the feces and urine, but a weekly analysis on a seven day composite of milk. Changes from one ration to another were made abruptly, but without any serious disturbances. Occasionally a sudden change caused a refusal of part of the ration for a day, but never longer than that.

Three animals were involved in these studies, two of which were pure bred Ayrshires of but moderate productive capacity,

[7] E. B. Hart and G. C. Humphrey: *ibid.*, xix, p. 127, 1914.

and the third a grade Holstein of large milk flow. In fact, for marked results in studies of this character animals of high production should be chosen. Preliminary feeding periods of ten days preceded the collection of the records. Animal No. 3 (Holstein) belonged to another group of experimental animals and had already been on the corn ration four weeks before the quantitative record was obtained. Consequently we have every confidence in the results of this record, although it was short.

No. 1 was with calf, but in the first three months of pregnancy. Nos. 2 and 3 were not with calf. It should be remembered that the daily storage of nitrogen by the fetus is small. A 75 pound calf containing when born 10 to 12 per cent of protein, would require the storage of but two grams of nitrogen per day, if the rate of storage was uniform for the entire period of gestation.

TABLE I.

Composition of rations.

	WEIGHT	NITROGEN	TOTAL N	DIGESTIBLE N CAL.	PRODUCTION THERMS	NUTRITIVE RATIO
	lbs.	*per cent*	*gm.*	*gm.*		
			Corn ration			
Corn meal	8	1.54	56.2	38.2	7.1	
Gluten feed	4	4.11	74.8	63.5	3.1	
Starch (corn)	1	0.04	0.2	0.2	1.0	
Corn stover	12	0.87	47.9	21.5	3.1	
Total	25		179.1	123.4	14.3	1:8
			Wheat ration			
Wheat	5.0	2.09	47.6	38.2	4.1	
Wheat gluten	1.2	11.40	62.1	62.1	1.2	
Starch (corn)	6.0	0.04	1.3	1.3	6.0	
Corn stover	12.0	0.87	47.9	21.5	3.1	
Total	24.2		158.9	123.1	14.4	1:8
			Milk protein ration			
Skim milk powder	3.0	6.00	81.5	81.5	3.0	
Casein	0.4	13.38	24.3	24.3	0.4	
Starch (corn)	8.0	0.04	1.8	1.8	8.0	
Corn stover	12.0	0.87	47.9	21.5	3.1	
Total	23.4		155.5	129.1	14.5	1:8

The composition of the rations used is given in Table I. The quantity of each ration consumed by the individual was kept constant during the several periods of observation involving the different rations. This was important, as it would allow the consumption of a constant quantity of roughage, the bulk of which determines so largely the quantity of nitrogen lost in the feces of an animal.

In Tables II, III, and IV are recorded the nitrogen balances and nitrogen elaborated in the milk by weekly periods. The figures represent the intake and outgo for the total seven days.

TABLE II.

Record of nitrogen balance, milk nitrogen, etc.

Animal 1 (Ayrshire)

DATE	N INTAKE	N FECES	N ABSORBED	N URINE	N MILK	N BALANCE
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Milk ration						
Dec. 22–28.......	997	445	552	262	283	+ 7
Dec. 29–Jan. 4...	1071	490	581	246	289	+ 46
Jan. 5–11.......	1071	426	645	202	274	+169
Wheat ration						
Jan. 12–18.......	1057	380	677	322	287	+ 68
Jan. 19–25.......	982	431	551	320	261	− 30
Jan. 26–Feb. 1...	1057	488	569	279	294	− 4
Feb. 2–8........	1057	502	555	274	291	− 10
Milk ration						
Feb. 9–15.......	1019	506	513	263	256	− 6
Feb. 16–22.......	1071	534	537	227	217	+ 93
Feb. 23–Mar. 1..	1071	566	505	181	251	+ 73
Mar. 2–8........	1071	571	500	236	252	+ 12
Corn ration						
Mar. 9–15.......	1148	537	611	292	267	+ 51
Mar. 16–22.......	1148	574	574	411	227	− 54
Mar. 23–31.......	1148	587	561	395	211	− 46

TABLE III.

Record of nitrogen balance, milk nitrogen, etc.

Animal 2 (Ayrshire)

DATE	N INTAKE	N FECES	N ABSORBED	N URINE	N MILK	N BALANCE
	gm.	gm.	gm.	gm.	gm.	gm.
Wheat ration						
Dec. 22–28	1092	494	598	414	276	− 92
Dec. 29–Jan. 4	953	404	549	464	227	−142
Milk ration						
Jan. 5–11	968	350	618	286	220	+112
Jan. 12–18	1071	459	612	250	260	+102
Jan. 19–25	1071	412	659	269	242	+148
Jan. 26–Feb. 1	1071	467	604	263	242	+ 99
Corn ration						
Feb. 2– 8	1148	504	644	303	295	+ 44
Feb. 9–15	1148	531	617	377	314	− 74
Feb. 16–22	1148	587	561	367	283	− 91
Feb. 23–Mar. 1	1148	579	569	343	282	− 56
Milk ration						
Mar. 2– 8	1020	470	550	292	297	− 39
Mar. 9–15	1071	518	553	231	266	+ 54
Mar. 16–22	1071	496	575	198	249	+128
Mar. 23–31	1071	448	623	184	256	+183

TABLE IV.

Record of nitrogen balance, milk nitrogen, etc.

Animal 3 (Holstein)

DATE	N INTAKE	N FECES	N ABSORBED	N URINE	N MILK	N BALANCE
	gm.	gm.	gm.	gm.	gm.	gm.
Corn ration						
Jan. 26–Feb. 1	1099	521	578	336	424	−182
Milk ration						
Feb. 2– 8	972	513	459	204	433	−178
Feb. 9–15	1022	535	487	184	369	− 66
Feb. 16–22	1022	467	555	197	354	+ 4
Feb. 23–Mar. 1	1022	489	533	199	342	− 8

In addition to the tables, charts are added showing the positive and negative nitrogen balances on these rations and the grams of nitrogen produced in the milk.

These data indicate very clearly a marked difference in the efficiency of the proteins used for continued milk production. A negative nitrogen balance always followed the use of the corn or wheat proteins in the concentration used, while a positive balance followed the use of the milk proteins. With Animal 3, where milk production was much larger than with either Animal 1 or 2, we did not expect to find a positive balance with the milk proteins. Nevertheless this animal on a 1:8 nutritive ratio with milk proteins approached the level of body maintenance with a high milk flow and milk protein production.

The really astonishing thing in the data is the influence of the forces of milk secretion in maintaining a yield of milk at the expense of tissue protein. Instead of finding a marked drop in the production of milk either in volume or concentration when these animals were on the corn or wheat ration, we found that they not only maintained the flow, but the amount of total solids, fat, and milk protein was also maintained. This was done, of course, at the expense of catabolized tissues. There was even some indication of a slight increase in the milk proteins elaborated during periods of negative nitrogen balance. This can be seen particularly in the curves for Animal 2. With this animal, during both wheat and corn protein periods with their negative nitrogen balances, the curve of milk protein building reached its highest level. Analogies to this condition are not wanting. It is now well known from the work of Eckles[8] that a cow freshening in a fat condition and then fed a sub-maintenance ration will tend to have a higher per cent of fat in the milk than under normal conditions of nutrition. Possibly in our own experiments the rapid tissue autolysis plus the food proteins slightly raised the mass of nitrogenous fragments in the blood, thereby creating suitable conditions for a somewhat larger protein elaboration by the mammary gland.

That negative nitrogen balances and sustained milk production could not be long continued is, of course, certain. Yet

[8] C. H. Eckles: *Missouri Agricultural Experiment Station Bulletins*, No. 100, 1912.

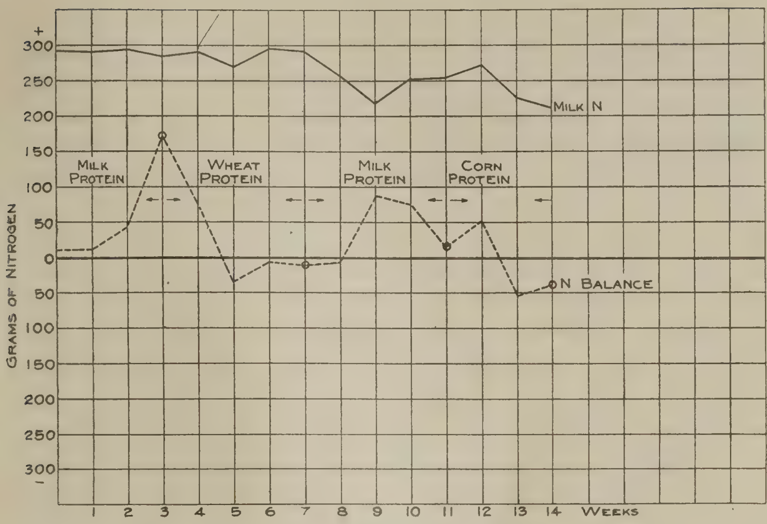

CHART 1. Animal 1. Showing the nitrogen balances with the different sources of protein and the milk nitrogen production.

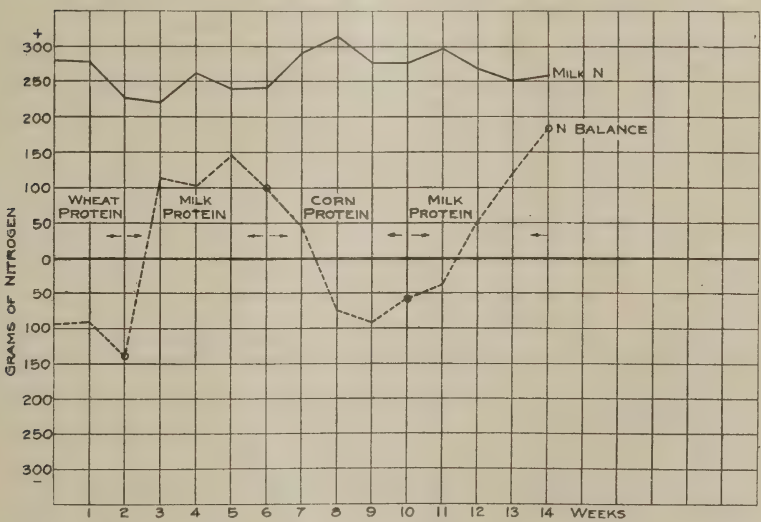

CHART 2. Animal 2. Showing the nitrogen balances with the different sources of protein and the milk nitrogen production.

for five weeks Animal 3 had continued to produce a daily flow of 35 pounds of milk with an average content of 10.5 per cent of solids and 2.7 per cent of total proteins on a nutritive ratio of 1:8 of corn proteins. She was probably in negative balance all this time, as evidenced by the record for a single week

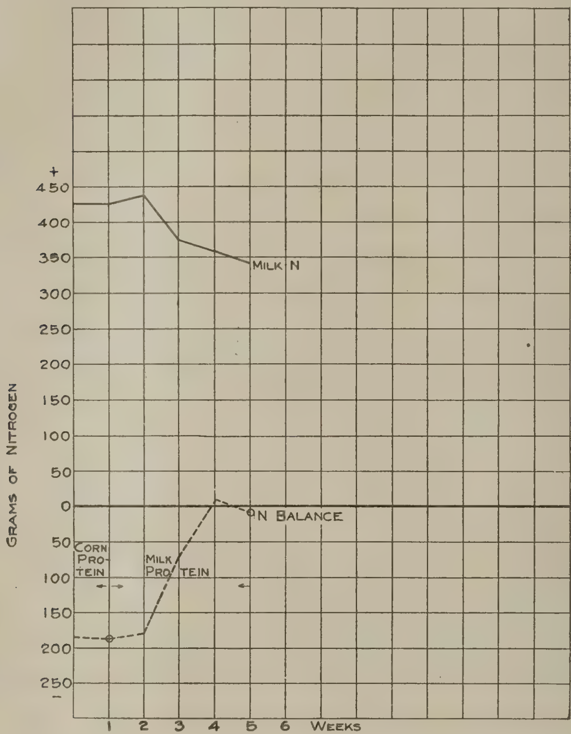

CHART 3. Animal 3. Showing the nitrogen balances with the different courses of protein and the milk nitrogen production. Note the high production of milk protein and the approach to a nitrogen balance with the milk proteins.

when the n trogen loss was 182 grams, or 2 5 pounds of protein. Casual observation showed that this animal was becoming emaciated by this constant withdrawal of protein material. "Milking flesh off the back" was in fact a reality. On the same nutritive ratio, but from milk proteins, this animal went on to a level of

nitrogen maintenance and continued high production What plane of intake of corn proteins would have been necessary for maintenance and continued high milk production remains to be determined, but it is apparent that such a method of investigation is the only one that will fix accurately the level of protein needed for milk production.

Records should preferably be made with high producing animals receiv ng a wide nutritive ratio. An mals in nitrogen equilibrium or showing positive nitrogen balances will not give the information desired. This is due to the fact that such records will not disclose the minimum amount of protein required for maintenance. Only when we begin to get a continued negative balance will we obtain information on the amount of protein needed for both maintenance and milk production. Starting from this negative balance and raising the protein intake to one giving nitrogen equilibrium should be the method of investigation of the problem of the protein requirement for milk production.

Attention should be called to the marked drop in urinary nitrogen in passing from either a wheat or corn protein ration to a milk protein ration. In some cases the decrease amounted to 40 per cent, indicating very much less waste and a more efficient utilization of the milk proteins. This is not to be attributed to the higher total nitrogen intake usually allowed on the corn or wheat protein ration. This higher intake was necessary in order that approximately equal quantities of digestible nitrogen should be available. For example, Animal 1 during the second milk protein period absorbed weekly, as an average for the period, 516 grams of nitrogen with a urinary elimination of 226 grams, while in the succeeding corn ration period she absorbed 582 grams, but eliminated in the urine 366 grams and also went into a negative balance. More striking perhaps and further illustrating the lower efficiency of the corn or wheat proteins is the case of Animal 2. On the milk protein ration (January 5th to February 1st) this animal absorbed weekly 623 grams of nitrogen and eliminated in the urine 267 grams, but was in a positive balance of 115 grams. On the corn ration (February 2d to March 1st) she absorbed weekly 588 grams, excreted in the urine 347 grams, and went into a negative balance of 45 grams.

With Animals 1 and 2—both moderate producers—nitrogen

balances always became negative on the corn and wheat proteins, but positive on the milk proteins. The storage of nitrogen occurring after a wheat or corn protein period could not have been indefinitely continued since these were mature animals, and it is known with certainty that no large storage of nitrogen under such conditions could take place. It probably does represent, however, the restoration of what was lost during the period of wheat or corn protein feeding and should be taken into account in measuring the comparative efficiency of the three sources of proteins. There was sometimes a lag in the appearance of a negative balance, as the animals were taken from the milk protein to the wheat or corn protein ration, which is probably to be explained on the basis of a moderate storage of nitrogen during the feeding of the highly efficient proteins, especially if this followed a period of intake of low efficiency proteins. There was also occasionally a lag in the appearance of positive balances, but these are to be attributed to the lower nitrogen intake following the change from a corn or wheat protein ration to the milk protein ration. The lower nitrogen intake was due to the failure of the animal to consume the full ration immediately after this change.

Efficiency of the proteins compared.

The fact that, over limited periods of time at least, no reduction in milk flow or milk protein elaboration may occur, although the animal is in negative nitrogen balance, makes it necessary to include the body protein formed or destroyed, in addition to the milk protein synthesized, in any calculation of the efficiency of proteins for milk production. In addition it appears probable that a percentage of efficiency can more correctly be based on the absorbed nitrogen than on the total nitrogen ingested. It is this method of calculation that will be presented, realizing that the results are more approximations than absolute accuracies, but that they do represent a degree of difference more easily appreciated when expressed in terms of percentage. The data are summarized for the total period of each ration and are recorded in Table V.

TABLE V.

Comparative efficiency for milk production of the proteins from corn, wheat, and milk.

Date	Ration	N absorbed	N in milk + tissue formed or destroyed	Efficiency
		gm.	gm.	per cent
Animal 1				
Dec. 22–Jan. 11	Milk	1778	1068	60.0
Jan. 12–Feb. 8	Wheat	2352	1157	49.1
Feb. 9–Mar. 8	Milk	2055	1148	55.8
Mar. 9–31	Corn	1746	656	37.5
Animal 2				
Dec. 22–Jan. 4	Wheat	1147	269	23.4
Jan. 5–Feb. 1	Milk	2493	1425	57.1
Feb. 2–Mar. 1	Corn	2391	997	41.5
Mar. 2–31	Milk	2301	1394	60.5
Animal 3				
Jan. 26–Feb. 1	Corn	578	242	41.8
Feb. 2–Mar. 1	Milk	2034	1260	61.9

The average percentage of efficiency with the three animals for the milk proteins was 59; corn 40; and wheat 36. Chart 4 graphically illustrates these differences.

Manifestly the average for the wheat proteins is probably somewhat too low, due to the very low efficiency of Animal 2 on this ration. More data are necessary to fix absolutely a safe figure for the wheat proteins. The averages for milk and corn are probably nearer the truth.

It should be observed that the average efficiency of milk proteins in milk production is not essentially different from their efficiency in growth as observed in this laboratory for swine and calves. It does appear, however, that there is a greater efficiency of the corn and wheat proteins for milk protein production than for growth. For growth in swine and calves the corn proteins showed a utilization of about 25 per cent, while the wheat proteins showed a utilization of 20 per cent. These differences in efficiency between growth and milk production may be due to

a certain specialized synthetic activity on the part of the mammary gland not possessed by the tissue cells. On the other hand, it is possible that the proteins of the roughage used, although small in amount, so supplemented the corn or wheat proteins as to raise slightly their efficiency. In addition, their higher utilization may be related to the amino-acid fragments liberated by catabolizing tissue. It is probably accurate to expect that the extra tissue catabolized during the periods of negative nitrogen balance would so supplement the ingested protein fragments from the corn or wheat as to make the latter appear, by the method of

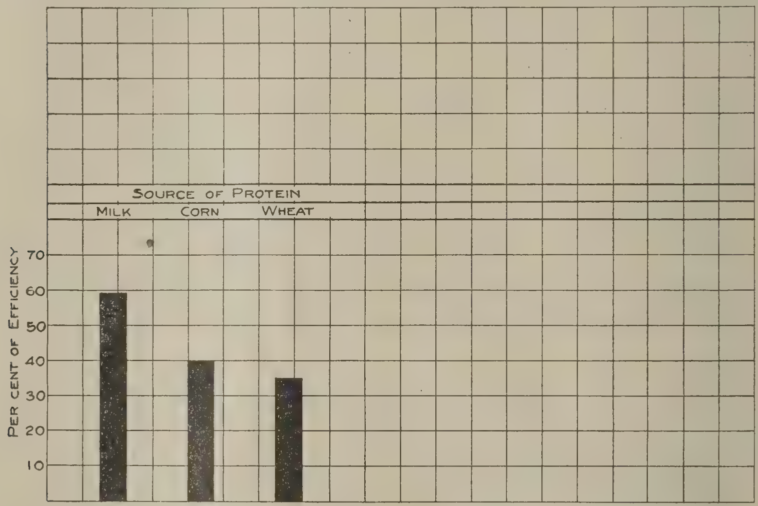

CHART 4. This shows the efficiency for milk production of the proteins studied when fed alone.

calculation, more efficient than they really are. This undoubtedly introduces a complexity and error in the figures deduced for efficiency, the magnitude of which cannot be estimated from the data. Further, in periods of positive nitrogen balance on the milk proteins, complexities are introduced. Not only was the mammary gland functioning, but in addition tissue building was taking place. It should not be assumed without proof that the two processes utilized building units with equal effect; consequently here again a slight error might be introduced in the calculations of the percentages of efficiency.

In any case, however, it is apparent that the synthetic powers of the mammary gland are not so pronounced as to make its efficiency independent of the nature of the proteins ingested with the feed.

SUMMARY.

Data are presented on the comparative efficiency for milk production of the proteins of milk, corn, and wheat grain.

On a nutritive ratio of 1:8, equivalent to about 7 per cent of digestible protein and 9 to 10 per cent of total protein, positive nitrogen balances were maintained with milk proteins for the production of as high as 35 pounds of milk per day, but negative balances resulted when the proteins were derived from the corn or wheat grain.

During the negative nitrogen balances increased tissue autolysis resulted, and for a brief time at least there was no decrease in the milk protein or milk solids elaborated.

The results indicate clearly that the *quality* of the proteins is an important factor in maintenance and production and that the synthetic powers of the mammary gland will not compensate for deficiencies in protein structure. The fact that negative balances were observed on the 1:8 nutritive ratio of corn or wheat proteins explains the results of experience which has wisely dictated a narrow nutritive ratio for milk production. The narrow ratio, of course, may not be so necessary when we learn with what efficiency the commercial protein concentrates may supplement a basal group of proteins like those of corn, oats, wheat, alfalfa, etc.

This opens anew the problem of the protein requirements for milk production which must be studied from the newer angle of protein structure.

It was found that milk proteins had an efficiency for milk production and tissue restoration of about 60 per cent, while the corn and wheat grain proteins showed an efficiency of 40 and 36 per cent respectively. These results were obtained under experimental conditions requiring a roughage of low nitrogen content.

Discussion of possible errors in the method of calculating the percentages of efficiency is introduced.

THE ESTIMATION OF PHOSPHORUS IN BIOLOGICAL MATERIAL, AND THE STANDARDIZATION OF SOLUTIONS OF MOLYBDENUM.

By A. E. TAYLOR and C. W. MILLER.

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(Received for publication, April 28, 1915.)

About a year ago we described two methods for the estimation of small quantities of phosphorus.[1] The first method is a modification of that of Neumann,[2] the phosphate being precipitated as the yellow phosphomolybdate and estimated by titration, after washing by decantation with the aid of the centrifuge. The results were stated to be somewhat too high because of the inclusion of molybdic oxide in the precipitate. Recently Raper[3] has described another modification of the same method, the yellow precipitate being dissolved in ammonium hydrate and the molybdenum precipitated as the lead salt, which is weighed. Raper states that when the yellow precipitate is formed in the presence of sulphuric acid oxide of molybdenum is also precipitated, a complication avoided by the substitution of nitric for sulphuric acid. This might be supposed to account for the too high values found by us, and we have therefore tried our method using nitric instead of sulphuric acid. Our original reagent consisted of a 10 per cent solution of ammonium molybdate in 2.5 per cent sulphuric acid (in our first publication this was erroneously given as 25 per cent). In the modification no acid was used in the molybdate, the plain water solution being added to the solution of phosphate containing 7 per cent of nitric acid. The results show that there actually is slightly less inclusion of the oxide of molybdenum with the use of nitric instead of sulphuric acid. More important, however, is the fact indicated

[1] A. E. Taylor and C. W. Miller: this *Journal*, xviii, p. 215, 1914.

[2] A. Neumann: *Ztschr. f. physiol. Chem.*, xxxvii, p. 133, 1902.

[3] H. S. Raper: *Biochem. Jour.*, viii, p. 650, 1914.

by precipitations under varying conditions, that oxide of molybdenum is included more and more in proportion to the excess of the ammonium molybdate present; in other words, the most accurate results are obtained with but a slight excess of ammonium molybdate present. Under this condition it makes little difference whether one operates with sulphuric or nitric acid. If a large excess of molybdate is present, the results are in any case much too high, though most markedly so with sulphuric acid. The following figures represent the results of numerous estimations with both acids in the presence respectively of slight and marked excess of ammonium molybdate. The phosphate solutions were Sörensen solutions containing either exactly 0.001 or 0.002 gram P.

Slight excess of reagent.

KNOWN 0.001 GM. P		KNOWN 0.002 GM. P	
Nitric acid	Sulphuric acid	Nitric acid	Sulphuric acid
0.00100	0.00104	0.00201	0.00205
0.00103	0.00102	0.00202	0.00206
0.00100	0.00101	0.00202	0.00204
0.00102	0.00097 (?)	0.00203	0.00202
		0.00201	0.00205
		0.00204	0.00200
		0.00203	0.00202
		0.00203	0.00203
			0.00202
			0.00204
			0.00205
			0.00204

The results are closer to the known content and somewhat less fluctuating with the nitric acid.

Notable excess of reagent.

KNOWN 0.001 GM. P		KNOWN 0.002 GM. P	
Nitric acid	Sulphuric acid	Nitric acid	Sulphuric acid
0.00104	0.00105	0.00208	0.00214
0.00106	0.00109	0.00210	0.00211
0.00108	0.00106	0.00209	0.00213
0.00106	0.00109	0.00210	0.00212

Obviously, excess of molybdate leads to marked inclusion of oxide of molybdenum, especially with sulphuric acid. This fact was overlooked by us before, as we had worked largely with standard phosphate solutions with which we had used but a slight excess of reagent. With the analysis of urine there was naturally no check.

Not only may oxide of molybdenum be precipitated as an inclusion with the yellow precipitate, but it may also sometimes be thrown out in our method by the use of too large amounts of absolute alcohol in the first two washings, a white film forming on the side of the tube near the top of the alcoholic layer. This white deposit should be removed by means of a rod and pledget of cotton after the clear supernatant liquid has been poured off. The following procedure gives satisfactory results.

Material containing about 2 mg. of phosphorus gives a desirable amount of precipitate. The ash from this amount of material (ashed as previously described by us) is dissolved in a small amount of dilute nitric acid and transferred to the 50 cc. centrifuge tube. The volume including the water used to rinse the dish in which the ashing was done, should not exceed 12 to 15 cc. About 2 grams of crystallized ammonium nitrate are then added in substance, followed by 10 to 12 drops of strong nitric acid. The mixture is then heated in the water bath. When hot, 2 cc. of a 10 per cent solution of ammonium molybdate are run in, the pipette being held centrally so that the molybdate does not fall on the walls of the tube. After a few moments the contents of the tube are shaken, then allowed to settle. After standing ten minutes, absolute alcohol not to exceed 5 cc. is carefully poured all around the sides of the tube, and the tube gently agitated by swinging so that the floating precipitate is scattered into the solution; over the latter is then floated 50 per cent alcohol and the alcohol is filled to the top of the tube. After the first centrifugation the supernatant liquid is poured off, 5 cc. of water are added, then 5 drops of strong nitric acid. The sides of the tube are now carefully rubbed clear with a rubber-capped rod, which is washed clean into the tube with a little water. The tube is then rotated for a few moments, 5 cc. of absolute alcohol are again carefully poured down the sides, the tube is gently agitated as before to scatter the floating precipitate, 50 per cent alcohol is again floated on and filled to the top, and the tube centrifugated. Thereafter

the yellow precipitate usually packs in the bottom of the tube and shows no further tendency to float on the surface. The fluid is poured off, about 10 cc. of 50 per cent alcohol added, the packed precipitate shaken loose, and the tube filled up with the alcohol. Five or six such washings, after the water wash, are usually sufficient, as the supernatant solution will then be found neutral.

In the original description we advised evaporation of the dissolved precipitate to dryness before titration. This we have since found may lead to aberrant results if the amount of alkali added for the solution of the precipitate be excessive. This is due to the fact that when oxide of molybdenum is evaporated to dryness in the presence of an excess of alkali or carbonate, oxide stages possessing a different amount of Mo to the molecule may be in part produced; as $Na_2Mo_2O_7$ instead of Na_2MoO_4. We now add a known volume of decinormal alkali, 25 cc. in the case of 0.002 of a gram of phosphorus, transfer the solution to a beaker with thorough washing of the tube into the beaker, place the latter on a water or sand bath until the volume is reduced to about 5 cc., when the excess of alkali is titrated.

The method of Raper in its entirety gives very excellent results, and may be advantageously used by anyone who does not possess a large centrifuge; although we find his method of preparing the yellow precipitate considerably more troublesome than our own. 0.001 gram P dissolved in 50 cc. of water and precipitated according to Raper gave almost correct values—0.00100 and 0.00101. Again, 1 cc. of two different urines gave:

METHOD OF RAPER	AUTHORS' METHOD
0.00040	0.00044
0.00063	0.00065

There seems to be no doubt that precipitation from dilute solution is accompanied with the very minimum of inclusion, a trace of which is not to be avoided when using the concentrated solutions necessary when the precipitate is to be washed in a 50 cc. centrifuge tube. If the slightly higher results of the tube method of preparing the yellow precipitate are allowed for, it will be found less time-consuming, as a large part of the operations involved requires little attention.

When the yellow precipitate has once been prepared and washed, the lead precipitation forms a very satisfactory means of completing the estimation. Thus the following values were obtained by dissolving in the tube the yellow precipitate prepared (according to our method) in 6 cc. of dilute ammonium hydrate; then transfer to a 250 cc. beaker, wash out the tube into the beaker using about 75 cc. of water, add 11 cc. of strong hydrochloric acid, then 10 cc. of a 4 per cent solution of lead acetate, and heat the mixture almost to boiling. In another beaker of 400 cc. capacity heat a mixture of 50 cc. of a solution of ammonium chloride (20 per cent) and 50 cc. of a solution of ammonium acetate (50 per cent). When both solutions are about boiling pour the first into the second, rinse the first beaker thoroughly with boiling water, and stir for a minute or so. Filter through a Gooch crucible, wash with boiling water until the washings no longer give a reaction with H_2S or an alkaline sulphide solution, dry, ignite, and weigh. 0.001 gram P gave:

0.00102
0.00102
0.00103
0.00100
0.00102

These correspond very well with the best results obtained by titration. An excellent feature of the lead precipitation is the exceptionally small plus or minus error involved. This will be appreciated when it is remembered that the factor for converting the $PbMoO_4$ to P is only 0.007.

We have not tried Raper's method with amounts of phosphorus as low as 0.0001, but if the data given by him are regularly reproducible, the method is more accurate than the second or colorimetric originally suggested by us. For many purposes, however, extraordinary accuracy is of no special advantage, since other errors in the investigation may be many times greater; under such conditions the colorimetric method is to be preferred on account of its much greater rapidity.

In our colorimetric method is involved the use of a known quantity of MoO_3. As it is difficult to purchase this substance in a pure condition, the amount of MoO_3 actually present in the commercial preparation employed should be determined.

Many methods of accomplishing this have been proposed, and, as is usually the case where it has been thought necessary to invent a large number of analytic methods, most of them are not very satisfactory. A summary of our experience with several of these might therefore be of interest.

We first thought that it might be possible to estimate the molybdenum by throwing it down as yellow precipitate with an excess of Na_2HPO_4, then washing and titrating as proposed by us for the estimation for phosphorus. The results were, however, worthless because, as mentioned by Krafft,[4] the yellow precipitate is quantitative only when an excess of the Na_2HPO_4 is avoided, although an excess of molybdate is quite permissible.

We then tried heating ammonium molybdate in the atmosphere so as to leave MoO_3, which was weighed as such. Results so obtained are usable, though the method is not satisfactory as it requires cautious heating and repeated weighings to arrive at approximately constant values.

If instead of heating in a crucible in the air, the molybdate be placed in a combustion boat and heated in a tube with a stream of hydrogen the product will be metallic molybdenum, which can be weighed as such. This method gave varying results, partly owing to a tendency to sublime, and is apparently worthless.

Estimation of molybdenum as mercurous molybdate[5] did not yield very satisfactory checks.

The process of reducing the molybdenum by means of zinc and sulphuric acid in a Jones' reductor from MoO_3 to Mo_2O_3, and then reoxidizing the latter by means of permanganate back to MoO_3, as given by Blair,[6] gives good results, with, however, an occasional wide variation. These latter would seem to be due to the uncertain amount of oxidation taking place in the receiving flask of the reducing apparatus. In the method as described by Blair the fact of slight oxidation is taken into consideration[7] by regarding the reduction product not as Mo_2O_3 but as $Mo_{24}O_{37}$. However, the amount of oxidation taking place does not seem to be at all times constant, which is already suggested by the

[4] F. Krafft: *Anorganische Chemie*, 4th edition, Leipsic, 1900, p. 435.

[5] F. P. Treadwell: *Analytical Chemistry*, 3d edition, London, 1914, ii, p. 285.

[6] A. A. Blair: *Analysis of Iron*, 7th edition, Philadelphia, 1912, p. 92

[7] See also Treadwell: *loc. cit.*, p. 639.

variations in the color of the liquid. We tried displacing the air in the receiving flask with CO_2 during the operation, but without any particular benefit as regards constancy of results. We finally tried the plan of placing in the reception flask a readily reducible solution which would immediately reoxidize the molybdenum as fast as it came down from the zinc, and yet which itself would not be so liable to reoxidation before titration. The following iron solution was found very satisfactory:

Iron alum	100 gm.
Sulphuric acid	25 cc.
Phosphoric acid	40 cc.
Water to make	1000 cc.

Heat and filter off from the white precipitate.

This solution is nearly the same as that given by Treadwell.[8] We also found it of advantage to use a reductor tube longer and not so wide as that of Jones, the inside dimensions of our tube being about 16 mm. by 550 mm. With this tube and using in the flask 25 cc. of the iron solution diluted with several times that volume of water very close titrations with decinormal permanganate were obtained. Thus six consecutive estimations, taking 100 mg. of commercial MoO_3, gave:

cc.
18.3
18.35
18.25
18.3
18.35
18.3

The objection to the above procedure is that the decinormal solution has a large molybdenum value, and the calculated results would be better with a weaker permanganate solution. For example, the above titrations, which are as close as one could expect to get, give the following percentages of MoO_3:

per cent
86.4
86.6
86.2
86.4
86.6
86.4

[8] Treadwell: *loc. cit.*, p. 640.

The calculation in this particular case is the following: The titration figure is multiplied by 1.006 to get the true value of the permanganate solution used; 0.4 cc., which is the blank given by the apparatus, is subtracted from the product, and the resulting figure is multiplied by 0.0048 to obtain MoO_3.

Of the lead precipitation methods we tried three: The Chatard[9] direct precipitation with lead acetate, giving good checks but usually low; the modification of this given by Merck;[10] and the Brearley and Ibbotson as described by Raper[3] as part of his method for phosphorus. The first two, in which the precipitation is direct, have the inconvenience that the precipitate comes out as a milky colloidal suspension which in our experience it is not easy to convert entirely into the granular form by boiling, as prescribed in these methods. The method given by Raper, on the other hand, produces at once a clean granular precipitate which can be filtered and washed with ease. The process as used by us was as follows:

To 100 mg. of MoO_3 add from 70 to 100 cc. of water, warm, and agitate until dissolved. To this solution, which will be more or less opalescent, add 10 cc. of concentrated hydrochloric acid, whereupon the solution should clear. Then add 10 cc. of a 4 per cent solution of lead acetate. A precipitation of white lead chloride may occur unless the solution is hot, but this is of no consequence, as it will redissolve upon heating. Heat to about the boiling point.

Meanwhile in another beaker heat to boiling a mixture of 50 cc. of ammonium acetate solution (50 per cent) and 50 cc. of ammonium chloride solution (20 per cent). When both solutions are hot pour the first into the second, rinse the first beaker thoroughly with boiling water, and stir for a minute or so. Filter on to a Gooch crucible, wash with boiling water until the washings give no further reaction with H_2S or an alkaline sulphide solution, dry, ignite, and weigh. The product is $PbMoO_4$, which multiplied by $0.3922 = MoO_3$.

Of all these various methods the last is probably the most satisfactory, with the titration with iron and permanganate the second choice.

[9] T. M. Chatard: *Chem. News*, xxiv, p. 175, 1871.
[10] G. Merck: *Chemical Reagents*, 2d edition, New York, 1914, p. 15.

BASAL METABOLISM AND BODY SURFACE.

A CONTRIBUTION TO THE NORMAL DATA.

By JAMES H. MEANS.[1]

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(Received for publication, April 15, 1915.)

It is rapidly becoming accepted that the study of the basal metabolism in disease is a field certain to yield valuable scientific and in all probability valuable clinical information. This being true, it becomes essential to have a reliable criterion for deciding whether or not the metabolism of any given individual may be considered normal. A comparison with the body weight is practically valueless, as has been well shown by Benedict.[2] Also the surface area as determined by Meeh's formula gives unsatisfactory results for people of abnormal states of nutrition (emaciated or obese).[3] The possibility that the ratio of basal metabolism to creatinine elimination might serve as a basis of comparison was considered in a paper by Palmer, Means, and Gamble.[4] A certain relationship was found in normal individuals, but since the appearance of this paper a far more satisfactory basis of comparison has become available. I refer to the method for determining body surface recently published by Du Bois.[5] The method has been fully described and needs no comment here.

The object of the present communication is merely to add to the normal data for basal metabolism per square meter of body surface as determined by this method.

[1] H. P. Walcott Fellow in Clinical Medicine, Harvard University.

[2] F. G. Benedict: Factors Affecting Basal Metabolism, this *Journal*, xx, p. 263, 1915.

[3] J. H. Means: Studies of the Basal Metabolism in Obesity and Pituitary Disease, *Jour. Med. Research*, xxxii, p. 121, 1915.

[4] W. W. Palmer, J. H. Means, and J. L. Gamble: Basal Metabolism and Creatinine Elimination, this *Journal*, xix, p. 239, 1914.

[5] D. Du Bois and E. F. Du Bois: The Measurement of the Surface Area of Adults, *Proc. Soc. Exper. Biol. and Med.*, xii, p. 16, 1914.

The metabolism determinations with the exception of that for Dr. P. D. W. all appeared in the paper on creatinine.[6] The

TABLE I.

Normal men.

SUBJECT	WEIGHT	HEIGHT	SURFACE AREA IN SQUARE METERS		CALORIES PER SQUARE METER AND HOUR		PERCENTAGE VARIATION FROM AVERAGE	
			Meeh	Du Bois	Meeh	Du Bois	Average 33.2 Meeh	Average 39.6 Du Bois
	kg.	*cm.*					*per cent*	*per cent*
Dr. W. W. P*........	93.9	187	2.541	2.118	29.9	36.8	−9.9	−7.1
Mr. H. L. H..........	62.0	172	1.927	1.648	34.1	39.8	+2.7	+0.5
Dr. W. S. W..........	73.8	177	2.164	1.847	31.9	37.5	−3.9	−5.3
Dr. L. W. H..........	68.4	169	2.057	1.703	33.9	40.9	+2.1	+3.3
Dr. P. H. P..........	77.2	172	2.179	1.806	31.0	37.4	−6.6	−5.6
Dr. J. H. M.**.......	70.7	175	2.103	1.794	33.2	39.4	0	−0.5
Dr. J. L. G...........	68.1	181	2.051	1.710	34.1	40.8	+2.7	+3.0
Dr. L. H. N.**.......	58.1	169	1.845	1.501	33.3	40.9	+0.3	+3.3
Dr. P. D. W.***.....	62.0	172	1.928	1.716	37.3	42.6	+12.3	+7.6
			Averages		33.2	39.6	4.5	4.0

* For this subject the average of all determinations given in a previous paper was taken.
** Some new determinations were obtained for these subjects and averaged with those previously published.
*** New subject.

TABLE II.

Normal women.

SUBJECT	WEIGHT	HEIGHT	SURFACE AREA IN SQUARE METERS		CALORIES PER SQUARE METER AND HOUR		PERCENTAGE VARIATION FROM AVERAGE	
			Meeh	Du Bois	Meeh	Du Bois	Average 29.9 Meeh	Average 38.2 Du Bois
	kg.	*cm.*					*per cent*	*per cent*
Miss M. A. H.........	57.9	157	1.841	1.454	32.4	41.0	+8.4	+7.3
Miss R. R............	70.9	169	2.107	1.737	32.5	39.5	+8.7	+3.4
Miss H...............	48.1	155	1.627	1.353	29.4	35.2	−1.7	−7.9
Miss D. L............	76.0	168	2.205	1.676	28.2	37.2	−5.7	−2.6
Miss F. M. R.........	77.7	166	2.240	1.737	30.4	39.3	+1.7	+2.9
Miss L. F. W.........	79.8	170	2.280	1.668	27.1	37.0	−9.4	−3.1
Miss R. Rob..........	67.5	170	2.039	1.558	29.6	38.6	−1.0	+1.0
			Averages		29.9	38.2	5.2	4.0

[6] Basal metabolism was calculated by indirect calorimetry from the oxygen absorption and R. Q., these being obtained with Benedict's Universal Respiration Apparatus. The subjects were all in the post-absorptive condition, lying flat and in complete muscular rest.

subjects, however, with two exceptions were available; so on learning of the Du Bois formula I had them all measured by that method. The results were very gratifying.

The results are shown in Tables I and II, the weight and height of the subjects and their surface areas by Meeh's and Du Bois'

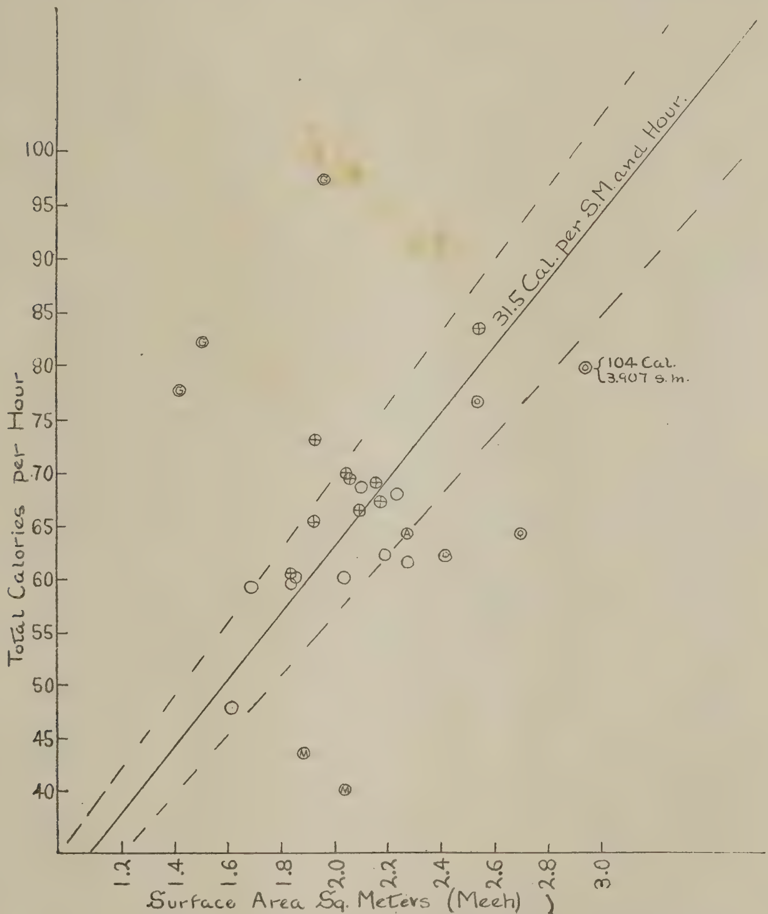

FIG. 1. Relation of metabolism to body surface as determined by Meeh's formula. Normal men are shown by circles containing crosses; normal women by plain circles; cases of obesity by circles containing O; cases of Graves' disease by circles containing G; myxedemas by circles containing M; and the case of acromegaly by a circle containing an A.

formulae, together with the metabolism per square meter and hour, by both methods for surface area. The results have also been plotted in Figures 1 and 2 (total calories per hour = ordinates; surface area in square meters = abscissae).

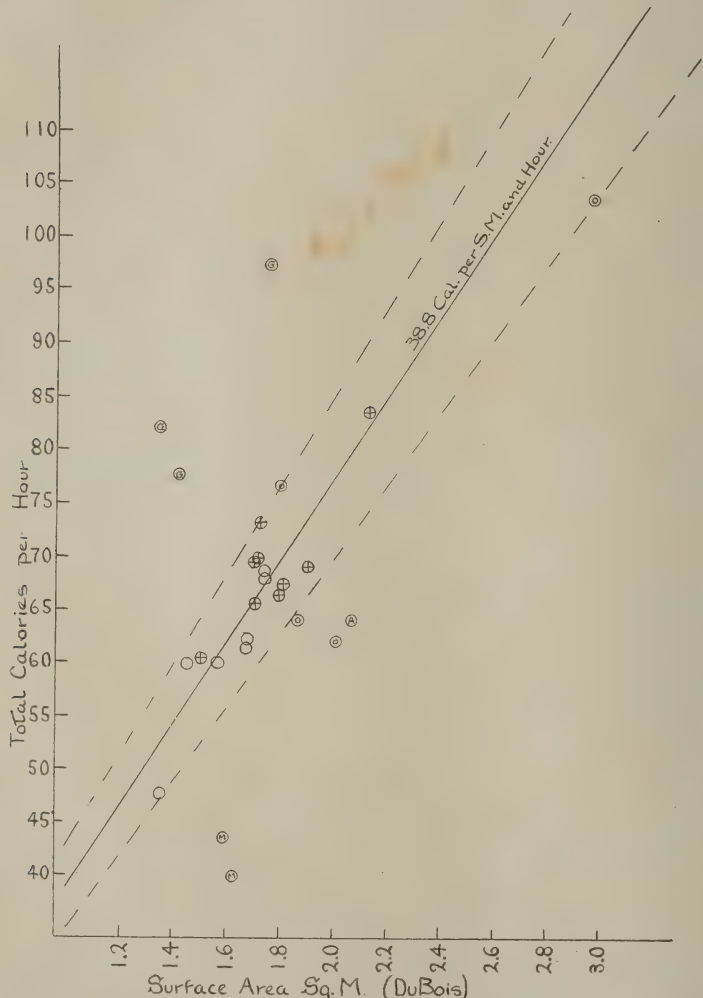

FIG. 2. Same as Figure 1. Body surface by Du Bois' formula.

DISCUSSION OF RESULTS.

In Figure 1 a diagonal representing a normal average of 31.5 calories per square meter and hour has been drawn, together with a $\pm$ 10 per cent deviation, represented by interrupted lines. This figure represents the Meeh body surface estimations. The average of 31.5 chosen is the mean of the average for men and the average for women in my series. It is somewhat lower than the figures 34.7 which Du Bois has obtained as an average for a large number of subjects, including his own and many of Benedict's.[7] It will be seen that out of eighteen normal subjects, two lie without and two on the edge of the $\pm$ 10 per cent (deviation from the average) zone.

Turning to Figure 2, however, which shows the Du Bois findings, we see that all of sixteen normal subjects lie within the zone and that the general appearance of the plot shows a somewhat closer grouping about the average than in Figure 1. The average taken for Figure 2 is 38.8 calories per square meter and hour, which is the mean of the average for men and the average for women in my series with the Du Bois formula.

A glance at these two figures will show a definite relationship between basal metabolism and body surface, and that the relationship is somewhat more striking when the Du Bois formula is used.

In a personal communication Dr. Du Bois tells me that he has obtained the figure 39.9 calories per square meter and hour as the normal average (Du Bois' formula). His basal metabolism determinations were made with the large respiration calorimeter, mine with the small Benedict Universal Respiration Apparatus; yet my average of 38.8 is within 3 per cent of his. I think this close agreement is very significant when one considers the entirely different types of apparatus used in making the metabolism determinations.

In my series the average deviation from the mean in the case of Meeh's formula was 4.5 per cent for men, 5.2 per cent for women, and in the case of Du Bois 4.0 per cent for both men and women. The extreme deviation was: Meeh, from + 12.3 per cent to -9.9 per cent for men, and from $+8.7$ per cent to -9.4

[7] E. F. Du Bois: Personal communication.

per cent for women; and with the Du Bois formula from +7.6 per cent to −7.1 per cent for men, and from +7.3 per cent to −7.9 per cent for women.

There is thus a smaller average as well as extreme variation when the Du Bois formula is used.

To emphasize the possible clinical importance of such studies certain pathological cases have also been plotted in Figures 1 and 2.

The four cases of obesity and one of acromegaly already reported by the writer,[8] together with three cases of Graves' disease and two of myxedema, will be reported fully in a subsequent paper. The three cases of Graves' disease lie well above the ± 10 per cent zone in both figures, the two myxedemas well below. The acromegalic is on the lower edge of the zone in the Meeh diagram, but well below in the Du Bois, and hence is undoubtedly pathological. In Figure 1 three of the obesities are below and one within the zone. In Figure 2 one is within, three on the edge of, and one well without the zone.

CONCLUSIONS.

The surface area calculated by both Meeh's and Du Bois' formulae, together with the basal metabolism per square meter of body surface, is given for sixteen normal persons.

The Du Bois formula gives figures for the metabolism which deviate less from the average.

The normal cases by the Du Bois formula all lie within a ± 10 per cent (from the average) zone.

Pathological cases, such as hypo- or hyperthyroidism, lie well without this zone, so that it seems reasonable to conclude that the surface area, especially when determined by the Du Bois formula, serves as a reasonable basis for detecting fundamental changes in metabolism.

My thanks are due to Miss L. A. Field, who made the measurements on all the women subjects. The men were measured by the writer.

[8] Means: *loc. cit.*

ON THE FORMATION OF FATS FROM PROTEINS IN THE EGGS OF FISH AND AMPHIBIANS.[1]

By J. F. McCLENDON.

(*From the Physiological Laboratory of the University of Minnesota, Minneapolis.*)

(Received for publication, April 27, 1915.)

That fats may be formed from proteins follows from the fact that carbohydrates may be formed from proteins and fats may be formed from carbohydrates. Metabolism experiments seem to show that fats are formed from proteins in the mammalian body under certain conditions, but this is not their chief source as was claimed by Voit. The fact that a lean meat diet is not fattening may be due to the production from the meat of acids which increase the hydrogen ion concentration of the blood, thus stimulating the respiratory center, increasing the heart rate, and causing increased oxidation of the food.

Beebe and Buxton observed an abundant production of fats from proteins by the action of *Bacillus pyocyaneus*. Other lower organisms are known to exert the same action, and Pflüger used this fact to explain the observation of Hofmann that the fat content of maggots fed on blood increased very much, as the blood was not sterile. Many observations on the increase in fat content of mammalian organs during autolysis and fatty degeneration may be vitiated by the presence of bacteria or by the use of micro-chemical methods for the detection of fat. Fatty acids may exist in combinations that are not detected by these methods, and the breaking up of these compounds by degeneration or autolysis may free them so that they can be detected.

As early as 1853 Burdach, in his inaugural dissertation at Königsberg, claimed that protein is changed to fat in the development of snail eggs, but he based this on small differences in ether extracts, and his work has not been confirmed.

[1] The trout eggs were given by the State Fish Commission, the *Cryptobranchus* eggs were bought out of the Research Fund of the Graduate School, and the larvae of the latter were given by Prof. Bertram G. Smith, to all of whom I wish to express my thanks.

Tangl and Farkas[2] observed an increase in the fat content of the developing eggs of European trout. The loss in dry weight during development was exceedingly small, and although CO_2 was eliminated, no nitrogen was lost. Ether extracts did not at all agree, either actually or relatively, with the results obtained by the saponification method of Liebermann and Székely,[3] which is a confirmation of the fact that the estimation of fats in tissues by extracting with ether is unreliable. They found no carbohydrate in the egg and concluded that fat is formed from a glycoprotein, since Walter[4] found a reducing substance in and formed an osazone from the hydrolytic cleavage products of the vitellin of carp eggs. On the contrary, Levene[5] found no reducing substance in the vitellin of cod eggs, and Hammarsten[6] found none in the vitellin of perch eggs. I was unable to obtain a Molisch reaction on the entire egg of the brook trout and it seems probable that the fat was formed from some other substance than a carbohydrate group.

Gortner[7] found an increase in the ether extract of the eggs of *Cryptobranchus allegheniensis* during development and concluded that there was an increase in fat. There was likewise an increase in the combined ether and alcohol extracts, and from my experiments it seems evident that practically all of his alcohol extract was soluble in ether.

The giant salamander.

The egg of the giant salamander, *Cryptobranchus allegheniensis*, is enclosed in a loose fitting gelatinous membrane of a glycoproteid. The space between the egg and the membrane is filled with a watery fluid containing 0.16 per cent of NaCl and other dissolved substances. The eggs were released by cutting the membrane with scissors. One egg, after partial removal of adhering fluid, weighed 0.128 gram. The average dry weight of one egg was 0.058 gram, which is the same to the third decimal place as found by Gortner.

Seven hundred eggs were used, for the most part in lots of 100. They were collected immediately after being laid in the Allegheny river and their development stopped by refrigeration during transit. A very few died and were thrown out and the remainder were dried *in vacuo*. The water vapor was pumped

[2] F. Tangl and K. Farkas: *Arch. f. Physiol.*, civ, p. 624, 1904.
[3] L. Liebermann and S. Székely: *ibid.*, lxxii, p. 360, 1898.
[4] G. Walter: *Ztschr. f. physiol. Chem.*, xv, p. 477, 1891.
[5] P. A. Levene: *ibid.*, xxxii, p. 281, 1901.
[6] O. Hammarsten: *Skan. Arch. f. Physiol.*, xvii, p. 113, 1905.
[7] R. A. Gortner: *Jour. Am. Chem. Soc.*, xxxvi, pt. ii, p. 1556, 1914.

out as fast as possible without causing sputtering, and the exhaustion continued until a vacuum of about 0.01 mm. was obtained and retained indefinitely. This method prevented any oxidation of unsaturated fatty acids.

The eggs were rapidly ground and extracted in a Soxhlet apparatus, fitted with a $CaCl_2$ U-tube, for one hundred and sixty-eight hours continuously with absolute alcohol that had been redistilled over sodium. They were then powdered and extracted twenty-four hours in a Soxhlet with ether that had been redistilled over sodium, but nothing more could be extracted after the alcohol treatment. Some oxidation must have occurred here but since the larvae were extracted the same way the results are comparative.

The alcohol extract was dried *in vacuo* to prevent oxidation, weighed and extracted with absolute ether in order to separate traces of salts that are extracted by the driest alcohol if the treatment is so prolonged.

All determinations were done in triplicate and the agreement was so close that only the average results will be given. For example, the ether-soluble substances were 34.8 per cent, 35.8 per cent, and 34.6 per cent of the dry weight of the eggs.

The non-volatile fatty acids in the extract were determined by the method of Kumagawa and Suto[8] and found to be 50 per cent of the weight of the ether-soluble substances. In another lot of eggs the lecithin was extracted with alcohol, dissolved in ether and precipitated by acetone. There was a considerable amount of it, but it could not be separated quantitatively. The low fatty acid content indicates the presence of more complex substances than lecithin or an unusually large amount of volatile fatty acids.

Two hundred larvae at the time of hatching were used, and treated in the same manner as the eggs. The larva twenty-four hours after hatching weighs about 0.21 gram, which is nearly double the weight of the egg. This increase is due to the absorption of water, since the dry weight of one larva is 0.057 gram, or one mg. less than the egg. Although my results agree closely with those of Gortner on this point, I think that the loss in weight

[8] M. Kumagawa and K. Suto: *Biochem. Ztschr.*, viii, p. 212, 1908.

during development is perhaps within the limit of error and consider this agreement more of a demonstration of the great uniformity of the material than an exact measure of the loss in weight.

The ether-soluble substances in the larvae were 37.8 per cent of the dry weight, and 50 per cent of the ether-soluble substances consisted of non-volatile fatty acids. We thus find an actual increase of 2.8 per cent in the ether-soluble substances when reckoned in terms of the total dry weight. This is an increase of 8 per cent in ether-soluble substances and the same increase in fatty acids.

Gortner extracted for forty-eight hours with ether followed by forty-eight hours with alcohol. His combined ether and alcohol extracts were 30.57 per cent of the eggs and 33.03 per cent of the larvae, showing that he had not extracted thoroughly, and yet the deficiency was uniform since he obtained an increase of 8 per cent in the "lipoids" during development.

These eggs consist chiefly of a vitellin, which I partially purified. It is soluble in 10 per cent sodium chloride and precipitated by saturation with ammonium sulphate but not by half saturation, or by saturation with magnesium sulphate. It is precipitated by $\frac{N}{100}$ hydrochloric acid and dissolves on neutralization. I had previously[9] learned the difficulty of removing by extraction the lecithin from vitellin or from eggs containing vitellins, a fact known to many investigators, and believe this difficulty is the cause of Gortner's low figures.

Since the whole egg gave no Molisch reaction, and a single hydrolysis gave no reducing substance, I concluded that there is no carbohydrate radical in the egg. The gelatinous membrane gives a strong Molisch reaction, but it consists largely of water, and the glycoproteid seems not to be absorbed at all by the embryo. It is therefore probable that the increase in fat is not due to the transformation of carbohydrate groups, but of proteins. The vitellin is the chief reserve material of this egg and out of it must be formed the various constituents of cells, some of which are fatty in nature. At the time of hatching a large part of the vitellin remains unchanged, and it is probable that

[9] J. F. McClendon: *Am. Jour. Physiol.*, xxv, p. 195 1909–10.

the increase in fat content continues until the yolk is all absorbed. I attempted to investigate this question, but the difficulty of keeping solid matter out of the mouths of the larvae together with various accidents has prevented the completion of this work.

The brook trout.

Eight hundred eggs and 200 young trout, *Savelinus fontinalis*, were obtained from the State Fish Hatchery in St. Paul. These were investigated in the same way as the salamander material, except that the egg membranes were not removed. The calculated weight of the membranes was added to the weight of the newly hatched fish, which had shed their membranes. The time necessary for complete extraction with absolute alcohol was shortened to sixty hours by keeping the condensed alcohol and extraction thimble at the boiling point.

The average weight of the egg is 0.062 gram and after drying, 0.017 gram. The loss in dry weight during development is about 2.5 per cent. The following table gives my results as compared with those of Tangl and Farkas, in per cent of the dry weight of the whole egg or young fish.

	EXTRACT			FATTY ACID		
	Egg	Young fish	Diff.	Egg	Young fish	Diff.
My data.........	23.4	24.7	1.3	16.17	17.16	0.99
Tangl	9.4	12.	2.6	20.5	21.8	1.3

I found an increase of 5.55 per cent in the ether-soluble substances and an increase of 5.57 per cent in the fatty acids during development. Tangl and Farkas observed 27.6 per cent increase in the ether extract and about 6.35 per cent increase in the fatty acids. Their method of extraction is well known to be inadequate and the fatty acids were titrated and not weighed. From the titration figures they calculated the fat by a formula of Liebermann for neutral mammalian fat, and obtained 21.4 per cent for the egg and 22.8 per cent for the young fish. Since mammalian fat contains about 95.7 per cent of higher fatty acids I multiplied their results by 0.957 in order to obtain the weight of these

acids. If the two species of trout are similar in chemical composition, the high figures may be explained by the hypothesis that the average molecular weight of the fatty acids is low. Such an assumption is not necessary to explain my data, however, since I found 69 per cent of the ether-soluble substances in the egg to consist of higher fatty acids, and this simulates closely the content of some tissues in ether-soluble substances. Lecithin contains about 70 per cent fatty acid, and the extract of normal mammalian liver contains 60–70 per cent of higher fatty acids.

The non-saponifiable substances soluble in petroleum ether formed 1.2 per cent of the dry weight of the eggs and 1.6 per cent of the young trout, showing an increase of 33 per cent. This extract crystallized into a solid mass largely composed of cholesterol crystals; but the cholesterol was not determined quantitatively. From my attempts with the trout and salamander eggs, I think one should start with at least 25 grams of dry material in determining the cholesterol quantitatively by the method of Kumagawa and Suto. The fatty acids of both species consisted chiefly of crystals at 20°; only a small fraction was liquid.

SUMMARY.

During the development of the *Cryptobranchus* egg there is an increase in the higher fatty acids of about 8 per cent. During the development of the brook trout egg this increase is about 5.6 per cent.

In these eggs the vitellin is the chief reserve material and it seems probable that it is transformed into fatty and other substances.

ON THE OXIDIZING POWER OF OXYHEMOGLOBIN AND ERYTHROCYTES.

By J. F. McCLENDON.

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(Received for publication, April 12, 1915.)

Bach and Chodat[1] found that some oxidases are mixtures of peroxidases and oxygenases (peroxides). Many persons believe that all animal oxidations are caused by mixtures of these two classes of enzymes, but this generalization may not yet seem justified. Warburg[2] found that in ground sea urchin eggs lecithin was oxidized in the presence of iron by O_2 (no CO_2 was produced). This oxidation may be performed *in vitro* on lecithin or linolein, and if any peroxides are present they are the oxidation products of the substance in question. It is the unsaturated fatty acid in these compounds that absorbs the oxygen, and Leathes[3] supposes that fatty acids are desaturated in the liver before they are burned in the body.

The blood pigments seem generally considered to be peroxidases; since in testing for their presence a peroxide and an oxidizable substance are added. Tests on blood pigments without the peroxide are very seldom tried, but I will show that oxyhemoglobin and methemoglobin will oxidize many leucobases without the addition of any other substance. It might seem more logical to consider oxyhemoglobin and methemoglobin as peroxides and hematin as a peroxidase; since the last will not act except on the addition of a peroxide. Peroxides, both organic and inorganic, are used commercially to accelerate the oxidation of linolein by O_2.

[1] A. Bach and R. Chodat: *Ber. d. deutsch. chem. Gesellsch.*, xxxvi, p. 600, 1903.

[2] O. Warburg and O. Meyerhof: *Arch. f. d. ges. Physiol.*, cxlviii, p. 295, 1912. O. Warburg: *Ergebn. d. Physiol.*, xiv, p. 254, 1914.

[3] J. B. Leathes: *The Fats*, 1st edition, London, 1914, pp. 112 and 113.

A greater or less degree of specificity is attributed to the oxidases, and it is even supposed that one oxidase causes only one step in the oxidation of a substance; as, for example, xanthooxidase which oxidizes xanthin to uric acid. On this hypothesis it seems hardly possible that the smallest microorganism could hold one molecule of every oxygenase and peroxidase together with their zymogens and kinases and arrangements to regulate their action, necessary to carry on its metabolism.

Living cells have an oxidizing action in the presence of O_2 and a reducing action in its absence. It does not seem to have been clearly demonstrated whether this reducing action is entirely due to oxygenases or partly due to split products of the food.

It has been especially emphasized by Battelli and Stern[4] that the respiration of the tissues rapidly decreases after the death of the animal, but continues at a slower rate for a long time. This slower respiration is apparently caused by the oxidases that may be extracted by water in the ordinary manner.

Warburg[5] showed that the rapid respiration characteristic of living cells is inseparable from structure. By grinding them in a steel ball mill he could completely destroy the respiration of some cells. Battelli and Stern,[6] using a Borrel mill (in which the cells are squeezed between revolving knives), claim that some respiration is left after destruction of cell structure, but they admit that the cell loses the power to oxidize citric, malic, and fumaric acids to CO_2 and H_2O on grinding in the Borrel mill for one minute.

Oxyhemoglobin.

Since the oxidizing power of oxyhemoglobin might be attributed to the presence of oxidases as impurities, thoroughly washed dog's erythrocytes were laked, the stroma was entirely removed, and the oxyhemoglobin recrystallized five times. Methemoglobin from the same source was recrystallized seven times. These two preparations showed about the same oxidizing power on the following substances:

[4] F. Battelli and L. Stern: *Biochem. Ztschr.*, lxvii, p. 443, 1914.

[5] Warburg: *Arch. f. d. ges. Physiol.*, cxlv, p. 277, 1912; *Ztschr. f. physiol. Chem.*, lxx, p. 413, 1910–11.

[6] Battelli and Stern: *loc. cit.*

α-Naphthol, aloin, and p-phenylene diamine were oxidized rapidly, whereas benzidine and guaiac were not. Vernon's[7] substrate (0.01 molecular α-naphthol and p-phenylene diamine in slightly alkaline solution) was oxidized with great rapidity. Goose oxyhemoglobin had the same action.

By means of a Duboscq colorimeter the oxidizing powers of solutions of the pure oxyhemoglobin and methemoglobin and dog's erythrocytes, all made up to the equivalent of 5 per cent blood, and acting on Vernon's substrate, were compared. It was found that the laked corpuscles did not oxidize the substrate faster than the purified blood pigments. By means of the following order of procedure the errors that would be caused by the difference in color of the oxyhemoglobin and methemoglobin were eliminated. Oxyhemoglobin was added to the methemoglobin solution, and methemoglobin to the oxyhemoglobin solution or blood just before reading.

The young erythrocytes of many animals oxidize foodstuffs, and blood charcoal oxidizes oxalic acid to CO_2 and H_2O.[8] From these two facts it might be deduced that adsorption and hence the colloidal state of the hemoglobin in the corpuscle is concerned in these oxidations. Many experiments to detect the production of CO_2 from lactic acid and sugars by means of blood charcoal or oxyhemoglobin proved negative. Incidentally it was found that lactic acid will drive CO_2 out of charcoal, and the latter had to be heated to redness and kept in a CO_2-free atmosphere until used.

Erythrocytes.

It was found that laked erythrocytes oxidize Vernon's substrate faster than the same concentration of unlaked erythrocytes. The simplest explanation is that the oxyhemoglobin in solution acts immediately on the substrate, whereas the blood pigment in the corpuscles cannot act until the substrate has reached it by diffusion. Hence the permeability of the corpuscles might affect the rate of oxidation by determining the rate of diffusion.

[7] H. M. Vernon: *Jour. Physiol.*, xlii, p. 402, 1911.
[8] Warburg: *Arch. f. d. ges Physiol.*, clv, p. 547, 1914.

Since laking has been shown by Stewart[9] to mean increase in permeability, and since it can be produced by electric condenser discharges, the attempt was made to produce varying degrees of permeability by means of induction shocks of varying intensity. The method consisted in measuring the electric conductivity of goose erythrocytes by the Kohlrausch method, using weak and strong induction shocks. The results were as follows:

EXPERIMENT	INDUCTION SHOCKS	ELECTRICAL RESISTANCE
1	Weak	1.08
	Strong	0.95
2	Weak	1.50
	Strong	1.38
3	Weak	1.15
	Strong	1.08
4	Weak	1.13
	Strong	1.08

The weak shocks were too weak to stimulate the nerves, and the strong shocks did not heat the erythrocytes measurably. The decreased resistance showed an increased permeability of the corpuscles, at least to ions, and yet they were not laked.

In order to determine whether induction shocks would increase the respiration of goose erythrocytes, they were suspended in Locke's solution (NaCl 8 grams, KCl 0.3 gram, $CaCl_2$ 0.3 gram, $NaHCO_3$ 1 gram, H_2O to make 1 liter) and placed in two similar vials with thermometers ground in for stoppers. Induction shocks were passed continuously through one vial by means of sealed-in platinum wires. In the experiments at 37°, a long capillary opening allowed expansion without error due to diffusion of oxygen. The erythrocytes were saturated with air at the beginning of the experiment, and at the end of the experiment were transferred to a closed flask fitted with a water manometer and shaken with air until equilibrium was again established. The CO_2 was held back by very dilute alkali. In some experiments the CO_2 produced was measured by dropping in tartaric acid without opening the flask (Barcroft),[10] and the CO_2 production was found to run parallel to the O_2 absorption. In other

[9] G. N. Stewart: *Jour. Pharm. and Exper. Therap.*, i, p. 49, 1909–10.
[10] J. Barcroft: *Ergebn. d. Physiol.*, vii, pp. 771 and 785, 1908.

experiments the whole operation was done in the manometric flask as outlined in the appendix.

EXPERIMENT	EXPOSURE	DIFFERENCE IN PRESSURE IN MM. H_2O		TEMPERATURE
		Control	Stimulated	
	min.			°*C.*
1	120	5	5	20
2	120	8	8	20
3	210	12	13	20
4	210	13	16	20
5	120	50	50	37
6	120	50	44	37

The table shows no increased using up of O_2 on electric stimulation with induction shocks that were found to increase the permeability, but since the oxygen and the oxidizable substances are already inside of the corpuscles we would hardly expect a more rapid oxidation on increasing the permeability. For this reason the experiments were repeated with the addition of an oxidizable substance (Vernon's substrate) to Locke's solution. The results are as follows, at 20°:

EXPERIMENT	EXPOSURE	FALL IN PRESSURE IN MM. H_2O	
		Control	Stimulated
	min.		
1	240	8	8
1	240	8	8
2	75	6	4
2	75	6	4
3	60	5	5
3	60	5	5
4	60	6	6
4	60	6	6
5	45	4	5
5	45	4	5
6	30	3	4
6	30	3	4

The experiments were done in duplicate and those that did not agree were thrown out. The different experiments cannot be

compared quantitatively because of the differences in the concentration of the erythrocyte suspension. It was found that if the exposure was long or the temperature was high (37°) there was some laking and the duplicates did not agree. Also, if the concentration of the erythrocytes was high the substrate was much reduced in concentration before the end of the experiment, and the results would be of no value. Hence the manometer differences were always small, and despite the most painstaking manipulation, cannot be regarded as conclusive.

Vernon's substrate, instead of increasing the respiration, seemed to exert an anesthetic action, as shown by the following experiments.

	REDUCTION IN PRESSURE IN MM. H_2O	
	Experiment 1	Experiment 2
Erythrocytes in Locke's solution......	8	6
Erythrocytes in substrate.............	6	4

Lillie[11] claimed that the oxidation of Vernon's substrate by frog's erythrocytes is increased by induction shocks, and my experiments either agree with his or are inconclusive. They do show, however, that electric stimulation does not increase the respiration of erythrocytes as much as it does that of muscle.

Appendix on technique.

In comparing readings on the manometer made at different times it is necessary to correct for barometric pressure or use some control on the changes in pressure. The following plan was found to be of service: Two water tanks were heated by means of the electric light circuit passed through the water from zinc plates. One tank was large and well insulated. The temperature regulator consisted essentially of a long straight bi-metal strip of invar and brass welded together (this can be had of makers of room thermostats) with platinum contacts that control the current of one dry cell. This in turn worked a telegraphic relay with large carbon contacts for controlling the heating current. The other tank was small and poorly insulated. It had a similar relay and heating arrangement, but the regulator was a copper tube filled with air and immersed in the water. One end was

[11] R. S. Lillie: this *Journal*, xv, p. 237, 1913.

closed and the other protruded out of the water and was connected with a Marey tambour, the lever of which was tipped with platinum and dipped in mercury. When the air expanded in the tube the lever rose out of the mercury and the heat was cut off. This tank might be called a barostat, since it compensated for changes in barometric pressure by changes in temperature. The two tanks were brought to the same temperature at the beginning of an experiment or first reading of the manometer, and all readings were taken in the barostat and the oxidations carried on in the thermostat. A cold water tap and overflow aided in keeping the tanks at low temperature. The ideal thermoregulator would be a tube of invar filled with mercury, but such tubes are not on the market.

ON THE COMPOSITION AND PHYSIOLOGICAL ACTIVITY OF THE PITUITARY BODY.

By FREDERIC FENGER.

(From the Research Laboratory in Organotherapeutics of Armour and Company, Chicago.)

(Received for publication, April 6, 1915.)

This investigation was conducted for the purpose of determining whether any seasonal variation exists in the pituitary body from cattle which furnish the raw material for the various pituitary preparations of the Armour Laboratory. If a seasonal variation exists, we should expect it to be most pronounced between glands obtained at the height of the outdoor season and those secured at the coldest period of the year. For this reason two summer and two winter months were chosen for collection. The summer glands were collected once a week during July and August, 1914, and the winter glands during January and February, 1915. In order to determine the difference, if any, in physiological activity between glands from various species of animals, pituitary bodies from hogs were also collected during the summer months.

A high percentage of the pituitary glands from beef contains colloidal material deposited in the space between the anterior and posterior lobes. It may be mentioned here that the colloid masses were found both in large and small glands indiscriminately, and that there seemed to be no relation between the size of the colloidal material and that of the gland. This colloidal material varies considerably in size, color, and consistency. The color ranges from an opalescent or milky white to dark amber. Other glands contain a considerable amount of liquid colloidal material. This varies from a thin, light colored opalescent liquid to a dark, heavy, semi-solid or gelatinous substance. It is very evident, therefore, that the solid colloidal material consists simply of the non-dialyzable substances present in the liquid colloidal secretion.

The amount of liquid between the lobes is often considerable and it was noticed in several instances that the posterior lobe had been reduced materially in thickness and the space between this and the anterior lobe completely filled with liquid colloidal material. In a few instances this material contained cluster-shaped masses of a white substance. Qualitative tests showed considerable CO_2, Ca, and P_2O_5, besides organic matter in these masses.

The method of collecting the glands was as follows: After the animals were slaughtered, the brain was removed and the pituitary body dissected from the sella turcica. The glands were carefully freed from adherent tissue, cleaned, weighed, and stored at freezing temperature until the entire lots had been collected. The posterior lobes were then dissected from the anterior lobes and the yields of both noted. The number of glands containing liquid and solid colloidal material was also recorded. The liquid and solid colloidal materials were kept separate and analyzed for comparative purposes. Portions of each lot of the fresh posterior lobes were reserved for physiological testing on the isolated uterus. The remaining glandular tissues were desiccated *in vacuo* at a low temperature, not exceeding 50° C., extracted with petroleum ether in a Soxhlet extractor, and powdered to pass a sixty mesh sieve. All the analytical work was done on this powdered substance.

The methods for determining the moisture, ash, and total phosphoric acid have been described elsewhere[1] in connection with investigations on the suprarenal gland.

In the tabulation below are given the total number of glands employed, the number of glands containing colloidal material, the maximum, minimum, and average weight of the fresh glands, the yield of posterior lobe from the fresh gland; also the moisture, petroleum-ether-soluble and yield of desiccated fat-free material both on the anterior and posterior lobes. On the desiccated, fat-free, powdered material, the moisture, ash, and total phosphoric acid were determined.

From the tabulated results it will be seen that the yield of posterior lobe from hog glands is about twice as high as that from corresponding beef glands. The anterior lobes from both species contain less moisture and petroleum-ether-soluble matter, and, therefore, give a larger yield of desiccated, fat-free material than

[1] A. Seidell and F. Fenger: *Bull. Hyg. Lab., U. S. P. H. and M.-H. S.*, No. 100, 1914.

	Fresh glands									Desiccated fat-free material		
	Total no. of glands	No. of glands containing colloidal material	Average weight of glands	Maximum weight of glands	Minimum weight of glands	Yield of posterior lobes	Moisture in fresh tissue	Petroleum-ether-soluble	Yield of desiccated fat-free material	Moisture	Ash	P_2O_5
			gm.	gm.	gm.	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Glands from cattle, collected July and Aug., 1914	797	80	2.7	5.0	2.0	9.5						
Glands from cattle, collected Jan. and Feb., 1915	915	83	2.4	5.0	1.0	8.4						
Hog glands, collected July and Aug., 1914	1866		0.33	0.8	0.1	18.2						
Anterior lobes from cattle, collected July and Aug., 1914							75.8	1.7	22.5	6.20	4.70	2.25
Anterior lobes from cattle, collected Jan. and Feb., 1915							76.5	1.9	21.6	5.80	4.50	2.48
Anterior lobes from hogs, collected July and Aug., 1914							74.3	2.3	23.4	3.30	6.20	3.40
Posterior lobes from cattle, collected July and Aug., 1914							79.6	2.5	17.9	3.30	5.20	2.45
Posterior lobes from cattle, collected Jan. and Feb., 1915							80.8	2.4	16.8	3.15	5.75	2.23
Posterior lobes from hogs, collected July and Aug., 1914							75.7	3.1	21.1	3.10	6.60	3.10

the posterior lobes. The posterior lobes in general contain a little more ash than the anterior lobes. No pronounced seasonal variation in the ash or phosphoric acid content could be demonstrated in this gland. Hog glands contain more ash and phosphoric acid both in the anterior and posterior lobes than beef glands. Approximately 10 per cent of beef glands contains masses of colloidal material secreted between the anterior and posterior lobes.

The physiological activity of the posterior lobe was determined by the isolated uterus method as outlined by Roth.[2] Besides the summer and winter glands from beef, two other lots were tested, one collected during the spring and summer of 1914, representing 15,000 animals from the Middle West, and another from southern and western cattle collected during the fall of 1914, and representing approximately 10,000 animals. All the seasons of the year are thus represented. The solutions, both from beef and hogs, were made to represent a 5 per cent solution of the fresh posterior lobe. The glands were finely minced, macerated with slightly acidulated isotonic salt solution, heated to boiling, and filtered. The clear liquid was filled into ampoules and sterilized. This liquid was used for comparison with the standard.

The standard was prepared by dissolving 0.1 gram of β-iminazolylethylamine hydrochloride in 100 cc. of distilled water. This liquid was filled into ampoules and sterilized at the same time and temperature as all the lots of pituitary liquid. The standard as well as the various pituitary liquids were made in duplicate. A 1:20,000,000 dilution of the standard in Locke's solution was maintained throughout the experiments. Various dilutions of the pituitary liquids were tried until the exact strength was found which matched the contraction produced by the standard. The results given here are averages of several determinations obtained in each case on three different days. The following dilutions of the original 5 per cent solutions of the posterior lobes were necessary to produce the same contractions as the 1:20,000,000 dilution of the standard.

[2] G. B. Roth: *ibid.*, No. 100, 1914.

Posterior lobes from beef, collected July and Aug., 1914	1:23,000
Posterior lobes from beef, collected Jan. and Feb., 1915	1:22,000
Posterior lobes from beef, collected spring and summer, 1914	1:22,000
Posterior lobes from beef, collected fall, 1914	1:23,000
Posterior lobes from hogs, collected July and Aug., 1914	1:22,000

It becomes apparent at once from the above figures that no distinct seasonal variation exists in the physiological activity of the posterior lobe of the pituitary body from cattle. It is also interesting to note that the strength seems to be the same both in cattle (herbivora) and hogs (omnivora).

The figures in the tabulation were obtained on the basis of a 5 per cent solution. On the basis of the fresh posterior lobe, therefore, the average strength of the active principle possessing uterine contracting power is 1:450,000, or approximately 1:45, when compared with pure crystalline β-iminazolylethylamine hydrochloride. Further work on the isolation of this active principle is in progress.

The two composite samples representing both liquid and solid colloidal material from beef glands were also tested. The strength was as follows on the basis of a 5 per cent solution:

Colloids from July and Aug. glands	1:600
Colloids from Jan. and Feb. glands	1:1000

When compared with the posterior lobe the physiological activity of this material is very slight. The material from the January and February glands shows a higher activity than that from the July and August glands. It was noticed that the winter glands contained comparatively much more colloidal liquid than the summer glands. As the solid colloidal material is insoluble in acidulated water, it is safe to assume that the slight uterine contracting power of the two composite samples is due to small quantities of soluble active principle from the posterior lobe dissolved in the colloidal liquid, and not to the colloidal material itself.

The total amount of colloidal material from the summer and winter glands was too small for chemical analysis. In order to get some more definite information regarding the composition

of these secretory products, a composite sample of solid colloidal material representing at least 30,000 cattle was analyzed. The moisture on the fresh material was 67.7 per cent. The dried substance contained:

	per cent
Moisture	5.95
Ash	3.85
Total phosphoric acid (P_2O_5)	0.58
Total nitrogen	13.98
Protein (nitrogen × 6.25)	87.40

The ash was of a reddish brown color and contained besides phosphorus, considerable quantities of iron and some calcium.

SUMMARY.

The posterior lobe of the pituitary body of the hog is twice as large in proportion to the weight of the entire gland as that in cattle.

The physiological activity of the posterior lobe, when determined according to the isolated uterus method, is practically the same for cattle (herbivora) as for hogs (omnivora).

No distinct seasonal variation in activity and chemical composition of the posterior lobe of the pituitary body exists in cattle.

Approximately 10 per cent of beef glands contains colloid masses secreted between the anterior and posterior lobes. This material is insoluble in acidulated water and does not possess any pronounced uterine contracting power.

THE DETERMINATION OF HIPPURIC AND BENZOIC ACIDS IN BLOOD AND TISSUE.

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(Received for publication, April 22, 1915.)

There are at present no methods for the estimation of benzoic and hippuric acids in blood of sufficient refinement to determine small amounts of these acids.

The method of Bunge and Schmiedeberg[1] and the modification of this method used by Kochs,[2] and the method used by Friedmann and Tachau[3] can not be used to detect small amounts of these acids since they depend upon the isolation of the crystalline substances with the usual losses of material remaining in solution in the mother liquors.

Folin and Flanders[4] have developed a quantitative method for the determination of benzoic acid in tomato ketchup, and the same investigators[5] have worked out a satisfactory method for the quantitative estimation of hippuric acid (total benzoic acid) in urine. In a previous communication by Kingsbury and Bell[6] it was shown that the first method, slightly modified, could be used for the determination of benzoic acid in rabbit urine, and that the second method was satisfactory without modification for the estimation of hippuric acid (total benzoic acid).

To apply these two methods to blood seemed an easy task, requiring only that the blood proteins be completely removed before the analyses were made. This is necessary, for proteins, treated according to the Folin-Flanders procedure for hippuric

[1] G. Bunge and O. Schmiedeberg: *Arch. f. exper. Path. u. Pharmakol.*, vi, p. 233, 1877.

[2] W. Kochs: *Arch. f. d. ges. Physiol.*, xx, p. 64, 1879.

[3] E. Friedmann and H. Tachau: *Biochem. Ztschr.*, xxxv, p. 88, 1911.

[4] O. Folin and F. F. Flanders: *Jour. Am. Chem. Soc.*, xxxiii, p. 1622, 1911.

[5] Folin and Flanders: this *Journal*, xi, p. 257, 1912.

[6] F. B. Kingsbury and E. T. Bell: *ibid.*, xx, p. 73, 1915.

acid, are hydrolyzed with the formation of benzoic acid. This was shown by Kingsbury and Bell[7] to be the case with the proteins, egg albumin, and casein.

The precipitation of blood proteins.

The blood is drawn from the carotid or femoral artery of the animal by means of a cannula into a weighed flask containing a weighed amount of 25 per cent magnesium sulphate solution, the volume of which is about one-quarter that of the blood to be drawn. The collecting Erlenmeyer flask has etched upon it 100 cc. graduation lines to facilitate the collection of the desired amount of blood. Care is taken to mix the blood and magnesium sulphate thoroughly to avoid clotting. When cool, the flask with the contained blood is weighed.

A weighed quantity, usually about 60 grams, of the magnesium sulphate-blood is centrifuged, the plasma poured into a small beaker, and the corpuscles hemolyzed by the addition of about 250 cc. of water. The plasma, hemolyzed corpuscles, and wash water are united, one drop of caprylic alcohol is added to prevent frothing, and the solution made up to the desired volume, 300 or 400 cc.

A 100 cc. aliquot of the dilute blood, contained in four centrifuge tubes, is treated with 2.00 grams of tannic acid dissolved in 10 to 12 cc. of water, and thoroughly mixed. By centrifuging for six to eight minutes, a water-clear, colorless filtrate is obtained. The precipitates in the four centrifuge tubes are washed four times with 300 to 350 cc. of water, using about 20 cc. for each tube each time. In order to wash the muddy precipitates thoroughly they must be carefully rubbed up with the wash water. The filtrate and washings combined amount to about 400 cc. This liquid may now be analyzed for benzoic or hippuric acids.

The determination of benzoic acid.

The filtrate and washings of the tannic acid precipitate, amounting to about 400 cc., are collected in two 500 cc. separatory funnels, each containing 110 grams of ammonium sulphate. The

[7] Kingsbury and Bell: *loc. cit.*

salt is dissolved by shaking, and the free benzoic acid in each separatory funnel determined as follows:

5 cc. of concentrated hydrochloric acid are added, and the solution is extracted with neutral, alcohol-free chloroform in one 50, one 35, and two 25 cc. portions. Each portion is drawn off into a second separatory funnel and the combined extracts are washed once with 100 cc. of a saturated sodium chloride solution containing 0.5 cc. of concentrated hydrochloric acid in 1000 cc. of solution. The washed extract of each separatory funnel is titrated against tenth normal sodium ethylate with phenolphthalein as indicator, and the titration figures are added.

The determination of hippuric acid (total benzoic acid).

A 100 cc. aliquot of the original diluted blood is precipitated with 2.00 grams of tannic acid and washed according to the method already described, and the combined filtrate and wash water are evaporated to dryness with 20 cc. of a 5 per cent sodium hydrate solution. The residue is treated exactly as in the Folin-Flanders procedure for the determination of hippuric acid.

Alternative method for the removal of blood proteins.

A second method for removing the proteins from diluted blood containing magnesium sulphate is described, as this method was used in obtaining some of the data in this paper and in the following one.

A 100 cc. aliquot of the diluted blood is boiled (without the addition of acid), and the clear, nearly colorless supernatant liquid filtered into an evaporating dish. The coagulum is washed with about 400 cc. of boiling water in small amounts, and the wash water evaporated on the steam bath with the original filtrate. When reduced to about 30 cc. in volume, the cooled filtrate is precipitated with 0.5 gram of tannic acid, and the small amount of precipitate resulting, washed with about 200 cc. of water. The filtrate, including wash water, is analyzed for benzoic acid or hippuric acid, according to the methods already described.

While not so convenient as the direct tannic acid precipitation method, the last method described is of more general appli-

cation and can be used with muscle and liver, which must be coagulated by heat in the process of extraction.

Tannic acid possesses the disadvantage of producing a titration blank when treated according to the Folin-Flanders procedure for hippuric acid. Tannic acid removes all material of a protein nature producing such blanks, and the final blank is due to the tannic acid alone. The acidified filtrates of heat-coagulated blood are very hard to extract with chloroform, but when further precipitated with tannic acid they are as easily extracted as any water solution. From this it is seen that the use of tannic acid is necessary in both analytical methods. Tannic acid has no effect whatever on the titration of free benzoic acid, as will be shown.

The filtrate of 50 cc. of 1 to 4 diluted rabbit blood, precipitated by boiling, was further precipitated with 0.20 gram of tannic acid and the filtrate analyzed according to the Folin-Flanders method for hippuric acid:

Titration........................0.10 cc. tenth normal ethylate
0.20 gram tannic acid alone, by this method:
Titration........................0.10 cc. tenth normal ethylate
0.50 gram tannic acid alone, by this method:
Titration........................0.15 cc. tenth normal ethylate

Filtrates of such heat-precipitated blood without further precipitation with tannic acid yielded titration blanks ranging from 0.20 to 0.40 cc. of tenth normal ethylate, depending on how completely the protein was removed by boiling.

While 0.20 gram of tannic acid was found to be enough in most cases to precipitate the proteins not removed by heat coagulation, it was customary to use 0.5 gram, which was sufficient in every case tried. In the analyses of liver and muscle recorded in the following paper, the filtrate from the heat-coagulated tissue extract was precipitated by tannic acid in 0.50 gram portions until the precipitation was shown to be complete. The filtrate and wash water of this precipitate were united and made up to the desired volume. Aliquot portions were taken for benzoic and hippuric acid determinations.

60 cc. of 1 to 4 diluted dog blood were precipitated directly with 1.0 gram of tannic acid, and the filtrate was analyzed by the Folin-Flanders method for hippuric acid:

Titration........................0.07 cc. tenth normal ethylate

In a similar analysis with the blood of another dog:

Titration........................0.08 cc. tenth normal ethylate

The same results were obtained with rabbit blood similarly treated. As is seen, the tannic acid titration blank obtained by the direct precipitation method is only 0.07 to 0.08 cc. of tenth normal ethylate for 1.0 gram of tannic acid as compared with 0.15 cc. for 0.50 gram of the latter when used in the alternative method.

It is evident that the amount of hippuric acid present in normal dog blood, if there is any at all, is too small to be detected by this procedure.

The titration blanks obtained by analyzing normal blood (of the dog, cat, rabbit, and ox) for free benzoic acid by the methods outlined were from 0.02 cc. to 0.05 cc. of tenth normal ethylate. These figures are within the limits of experimental error.

The use of chloroform as the solvent for benzoic acid in the presence of hippuric acid has one disadvantage: it dissolves a small amount of hippuric acid, and this is titrated with the benzoic acid, making the figure for the latter too large. This source of error is not a serious one. Titration blanks for the presence of hippuric acid were determined under exactly the same conditions as those under which the determinations for benzoic acid were made. The results are shown in Table I.

TABLE I.

Hippuric acid present	Hippuric acid extracted: cc. tenth normal ethylate	
gm.		
0.300	0.25	Average results of several determinations.
0.200	0.20	
0.100	0.20	
0.050	0.20	
0.010	0.05	
0.005	0.05	

Raiziss and Dubin[8] have recently suggested the use of toluene for the extraction of benzoic acid from urine. Their finding that

[8] G. W. Raiziss and H. Dubin: this *Journal*, xx, p. 125, 1915.

toluene does not extract a titratable amount of hippuric acid has been confirmed. Toluene has been tried as the solvent for benzoic acid, but it was found that the method of procedure would have to be modified in order to use it successfully. Since the amounts of hippuric acid extractable with chloroform in the benzoic acid procedure had been worked out before the article of Raiziss and Dubin appeared, and since the methods, as outlined, were being used to determine hippuric and benzoic acids in the dog experiments described in the following paper, it was thought advisable to continue the use of chloroform until the applicability of toluene could be more thoroughly investigated.

In order to determine the accuracy of these methods, hippuric and benzoic acids, separately and together, were added to diluted, hemolyzed blood and the latter was analyzed. The results are shown in Tables II, III, and IV. It seems probable that the small amounts of these acids not recovered are held back by the protein precipitates. Prolonged washing of these precipitates did not, however, increase the yields of benzoic and hippuric acids.

TABLE II.

Recovery of benzoic acid added to blood.

BLOOD	BENZOIC ACID EQUIVALENT TO Na BENZOATE ADDED	BENZOIC ACID FOUND	RECOVERED
	gm.	*gm.*	*per cent*
Rabbit			
1:10 dilution			
50 cc.	0.1471	0.1423	
"	0.1471	0.1435	98.1
"	0.1471	0.1469	
Dog			
1:3 dilution			
50 cc.	0.1471	0.1463	
"	0.1471	0.1457	99.0
"	0.1471	0.1446	
Dog			
1:4 dilution			
50 cc.	0.1471	0.1423	
"	0.1471	0.1418	97.2
"	0.1471	0.1446	
Rabbit			
1:4 dilution			
25 cc.	0.1471	0.1430	97.2

TABLE III.

Recovery of hippuric acid added to blood.

BLOOD	HIPPURIC ACID (NEUTRALIZED WITH NaOH) ADDED	HIPPURIC ACID RECOVERED	RECOVERED
	gm.	*gm.*	*per cent*
Rabbit 1:5 dilution			
50 cc.	0.1260	0.1218	96.8
"	0.1260	0.1228	97.6
Rabbit 1:10 dilution			
50 cc.	0.1770	0.1705	96.4
"	0.1770	0.1737	98.8

TABLE IV.

Recovery of benzoic and hippuric acids added to blood.

(Rabbit blood diluted 1 to 4.)
50 cc.

BENZOIC ACID EQUIVALENT TO Na BENZOATE ADDED	HIPPURIC ACID ADDED (NEUTRALIZED WITH NaOH)	BENZOIC ACID EQUIVALENT TO TOTAL BENZOIC ACID ADDED	BENZOIC ACID RECOVERED	HIPPURIC ACID RECOVERED (BY DIFFERENCE)	BENZOIC ACID EQUIVALENT TO TOTAL BENZOIC ACID RECOVERED	BENZOIC ACID RECOVERED	HIPPURIC ACID RECOVERED	TOTAL BENZOIC ACID RECOVERED
gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
0.1471	0.1893	0.2760	0.1435	0.1695	0.2591	97.6	89.6	94.0
0.1471	0.1893	0.2760			0.2585			93.7
0.1471	0.1413	0.2435			0.2272			93.4

SUMMARY.

Quantitative methods based on those of Folin and Flanders for the determination of hippuric and benzoic acids have been applied to blood and tissue analysis. By means of these methods small quantities of these two acids can now be estimated. The practical application of these methods is demonstrated in the following paper.

THE SYNTHESIS OF HIPPURIC ACID IN NEPHRECTOMIZED DOGS.

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At the present time it is widely believed that in the dog the kidneys have the special function of synthesizing hippuric acid. This view rests mainly on the classical experiments of Bunge and Schmiedeberg. The object of this investigation is to study this problem by means of the more accurate analytical methods which are now available.

Bunge and Schmiedeberg[1] first attacked the problem by ligaturing all the blood vessels of the liver, and then injecting sodium benzoate and glycocoll into the venous circulation. This experiment was tried with two dogs which were killed fifty-five and fifty minutes respectively after the injection. In the first experiment they found a small amount of hippuric acid in the blood; but in the second experiment they were unable to find it with certainty.

Two of their other experiments have a direct bearing on this investigation. In these experiments they ligatured the renal vessels of both kidneys securely, and then injected sodium benzoate and glycocoll intravenously. The first dog, killed at the end of one and one-fourth hours, did not show any hippuric acid in the blood, although benzoic acid was present in large amounts. The second dog was killed two and one-half hours after the injection. No trace of hippuric acid was found in the blood, liver, or muscles; but benzoic acid was present in all three tissues.

In another dog both ureters were ligatured, and sodium benzoate and glycocoll were injected intravenously. Blood drawn two and one-half hours later contained appreciable amounts of both hippuric and benzoic acids.

By perfusing kidneys of dogs with defibrinated dog blood, containing sodium benzoate and glycocoll, these investigators found that hippuric acid was formed.

On the basis of these experiments Bunge and Schmiedeberg concluded that the kidney is the only organ in the dog in which hippuric acid is synthesized.

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[1] G. Bunge and O. Schmiedeberg: *Arch. f. exper. Path. u. Pharmakol.*, vi, p. 233, 1877.

W. Kochs,[2] and Bashford and Cramer[3] repeated Bunge and Schmiedeberg's perfusion experiments and confirmed them.

The analytical methods employed in our experiments are described in detail by Kingsbury[4] in an article which appears in this number of the Journal.

EXPERIMENTS.

Dog A, male; weight 15,470 gm. This animal was given water but no food during the forty-eight hours preceding the operation. 1 gm. of morphine was injected subcutaneously, and one hour later both kidneys were removed aseptically under ether anesthesia. The wound was closed and the animal was left in a warm room without food. The next day it seemed in good condition. Twenty-four hours after the nephrectomy the animal was etherized and 365.85 gm. of blood were drawn by cannula from the femoral artery into 82.77 gm. of 25 per cent magnesium sulphate solution.

The liver was removed immediately after the animal had been bled to death, separated from the gall bladder, and washed in water to remove the surplus blood. The surface moisture was then removed by filter paper. The weight of the liver was 360.4 gm. A piece of the right psoas muscle weighing 38.1 gm. was removed for analysis.

61.1 gm. of magnesium sulphate blood, corresponding to 49.81 gm. of undiluted blood, 50.0 gm. of liver, and 16.5 gm. of muscle were analyzed, according to the method referred to above, to determine the blanks in the benzoic and hippuric acid (total benzoic acid) methods. The following table shows the results.

TABLE I.

Dog A. Control animal.

ORGAN OR TISSUE	AMOUNT OF ORGAN OR TISSUE ON WHICH THE BLANK WAS MADE	HIPPURIC ACID (TOTAL BENZOIC ACID) METHOD. BLANK IN CC. $\frac{N}{10}$ ETHYLATE	FREE BENZOIC ACID METHOD. BLANK IN CC. $\frac{N}{10}$ ETHYLATE
	gm.	*cc.*	*cc.*
Blood	15.2	0.05	
Blood	7.75		0.05
Liver	12.5	0.05	
Liver	12.5		0.05
Muscle	6.6	0.05	
Muscle	6.6		0.02

[2] W. Kochs: *Arch. f.d. ges. Physiol.*, xx, p. 64, 1879.

[3] E. Bashford and W. Cramer: *Ztschr. f. physiol. Chem.*, xxxv, p. 324, 1902.

[4] F. B. Kingsbury: this *Journal*, xxi, p. 289, 1915.

These blanks were all within the limit of experimental error, but it was thought best to consider them in the analyses of the blood, liver, and muscle of the nephrectomized animals.

Dog B, male; weight 12,600 gm. The treatment preliminary to operation was the same as in the preceding experiment. Both kidneys were removed. Immediately after the nephrectomy the dog was injected subcutaneously with 60 cc. of a sodium benzoate solution equivalent to 5.031 gm. of benzoic acid, and 30.9 cc. of a glycocoll solution containing 3.09 gm. glycocoll, an amount approximately equivalent to the benzoic acid used. Twenty-four hours later the animal was etherized and 354.52 gm. of blood were drawn from the femoral artery into 55.33 gm. of 25 per cent magnesium sulphate solution.

68.14 gm. of magnesium sulphate blood, corresponding to 55.30 gm. of blood, were analyzed for hippuric and benzoic acids, the blanks obtained with the control animal, Dog A, being taken into consideration

The hippuric acid (total benzoic acid) titration was 1.35 cc. $\frac{N}{10}$ sodium ethylate for 31.65 gm. of blood. After subtracting the tannic acid titration blank[5] of 0.15 cc. and the blank of 0.05 cc. determined in the blood of the control animal, the titration was 1.15 cc., the equivalent of 0.01404 gm. of benzoic acid.

The free benzoic acid titration for 20.63 gm. of blood was 0.59 cc. of $\frac{N}{10}$ sodium ethylate. After subtracting 0.05 cc. as the equivalent of the maximum amount of hippuric acid that could have been extracted with the benzoic acid, and 0.05 cc., the blank obtained with the control animal, the titration figure is 0.49 cc., which is equivalent to 0.00598 gm. of free benzoic acid.

The results expressed in gm. per 100 gm. of blood are:

Total benzoic acid found	0.0444 gm. as benzoic acid.
Free benzoic acid found	0.0290 gm. as benzoic acid.
Hippuric acid by difference	0.0226 gm. as hippuric acid.

Estimating the total quantity of blood in the animal as approximately one-thirteenth of the body weight, we obtain about 0.23 of a gram of hippuric acid as the quantity present in the entire blood.

Dog C, female; weight 7050 gm. The preliminary treatment was the same as in the preceding experiments. Both kidneys were removed. Immediately after the operation 30 cc. of sodium benzoate solution equivalent to 2.516 gm. of benzoic acid and 15.5 cc. of glycocoll solution containing 1.55 gm. of glycocoll were injected into the femoral vein. Three hours later 239.39 gm. of blood were drawn from the femoral artery for analysis.

[5] Kingsbury: *loc. cit.*

Analysis of blood.

Found in 25.16 gm. of blood	0.01822 gm. of total benzoic acid.
Found in 25.16 gm. of blood	0.01322 gm. of free benzoic acid.

In 100 gm. of blood:

Total benzoic acid	0.0725 gm. as benzoic acid.
Free benzoic acid	0.0525 gm. as benzoic acid.
Hippuric acid	0.0293 gm. as hippuric acid.

Estimated for the entire blood the amount of hippuric acid is roughly 0.16 gram.

As was mentioned above, Bunge and Schmiedeberg, in two experiments essentially the same as the one just described, were unable to find even a trace of hippuric acid in the blood.

Dog D, female; weight 14,745 gm. The preliminary treatment was the same as in the preceding experiments. Both kidneys were removed, and immediately after the operation 120 cc. of a sodium benzoate solution equivalent to 10.06 gm. of benzoic acid, and 60 cc. of a glycocoll solution equivalent to 6.0 gm. of glycocoll were injected subcutaneously. Twenty-four hours after the operation, 300.58 gm. of blood were drawn from the femoral artery for analysis. The animal was then bled to death, and the liver and a piece of the right psoas muscle, weighing 37.91 gm., were removed for analysis. After removal of the gall bladder the liver weighed 384.80 gm.

Analyses.

Found in 12.21 gm. of blood	0.01265 gm. of total benzoic acid.
Found in 12.21 gm. of blood	0.00718 gm. of free benzoic acid.
Found in 24.93 gm. of liver	0.0535 gm. of total benzoic acid.
Found in 24.93 gm. of liver	0.0251 gm. of free benzoic acid.
Found in 37.91 gm. of muscle	0.00455 gm. of total benzoic acid.
Found in 37.91 gm. of muscle	0.00171 gm. of free benzoic acid.

The results calculated for 100 grams of blood and for the entire liver are given in Table II.

TABLE II.

	100 GM. BLOOD	ENTIRE LIVER (384.8 GM.)	PSOAS MUSCLE (37.91 GM.)
	gm.	*gm.*	*gm.*
Total benzoic acid	0.1036	0.8260	0.0046
Free benzoic acid	0.0588	0.3875	0.0017
Hippuric acid	0.0658	0.6435	0.0042

Estimated for the entire blood the amount of hippuric acid is roughly 0.7 gram. The amount of hippuric acid in 1 gram of liver is about two and one-half times the amount in 1 gram of blood.

The extract of 249.3 gm. of this dog's liver was freed from protein by boiling and by the subsequent tannic acid precipitation, and then made up to 1000 cc. Two 100 cc. portions were used for the total benzoic and free benzoic acid determinations. 600 cc. of the remaining 800 cc. of solution were acidified and repeatedly extracted with ether. The combined ether extracts were evaporated to dryness and the residue was dissolved in water by rendering it alkaline. This solution was again acidified and extracted with toluene in four portions, completely removing benzoic acid which was identified as such, and leaving hippuric acid in the water layer from which it was crystallized, boiled with bone-black, recrystallized, and positively identified as hippuric acid.

In both experiments the subcutaneous areas, where the benzoic acid and glycocoll were injected, were found very edematous at the autopsy, showing that by no means all of the fluids injected had been absorbed.

The relatively large amount of hippuric acid found in the liver of a nephrectomized dog after injections of glycocoll and sodium benzoate suggests the hypothesis that the liver of the dog, like that of the rabbit (Friedmann and Tachau[6]) may possess this synthetic power.

SUMMARY AND CONCLUSIONS.

The foregoing experiments prove that considerable amounts of hippuric acid may be synthesized in nephrectomized dogs. It therefore follows that the kidney is not the only organ in the dog which may effect this synthesis.

We are indebted to Mr. R. A. Johnson, of the Department of Pathology, for assistance in the surgical operations.

[6] E. Friedmann and H. Tachau: *Biochem. Ztschr.*, xxxv, p. 88, 1911.

STUDIES ON THE REDUCTASE OF LIVER AND KIDNEY.

III. THE INFLUENCE OF HEAT, LIGHT, AND RADIUM RADIATIONS ON THE ACTIVITY OF REDUCTASE.[1]

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I. INTRODUCTION.

The influence of different kinds of radiant energy on enzymes has been largely investigated during the past few years. It is well known that enzymes are highly susceptible to the action of heat, and that when enzymes in aqueous solution are heated above certain temperatures a partial permanent inhibition of their activity occurs. The extent of this inhibition of the enzyme increases as the temperature is raised, and, finally, if the temperature to which the enzyme is submitted is high enough, permanent inhibition is complete,—the activity of the enzyme is completely destroyed. The lowest temperature at which complete permanent inhibition or destruction occurs is different for different enzymes. This total destruction is supposed to be due to a suspension of the active surface force of the enzyme by coagulation. As up to the present no case is known of the regeneration of the activity of an enzyme that has been completely destroyed by heat, such destruction may, therefore, be regarded as irreversible inactivation. Other types of radiations exhibit different actions towards different enzymes. The action of light rays varies not only with the enzyme, but also with the part of the

[1] The expenses of this research were met by a grant to one of us (D. F. H.) from the Government Grants Committee of the Royal Society of London, which is hereby gratefully acknowledged.

spectrum employed. For example, Green found[2] that the ultra-violet rays exerted a strong destructive action on diastase; the green rays acted in the same way; on the other hand, other portions of the spectrum were found to activate the enzyme at first, especially rays in the red, orange, and blue. Ultimately these rays caused destruction of the enzyme. The different diastases were found to behave differently; the malt extract was destroyed up to 68 per cent, while the diastases of saliva and leaves were destroyed up to 45 per cent and 8 per cent, respectively. The destructive action of radium radiations on emulsin and trypsin has been investigated by Henri and Mayer,[3] and the activation of animal amylase by radium emanation by Loewenthal and Wohlgemuth.[4] Richter and Gerhartz have found[5] that X-rays have no appreciable influence on the activity of enzymes.

In a previous communication[6] it was shown that between 10° and 40° the temperature coefficient of the reduction of oxyhemoglobin to the one-banded condition by reductase was approximately 2, while above the latter temperature the temperature coefficient rapidly decreased as the temperature at which the reduction took place was raised. It was pointed out that this decrease in the temperature coefficient was probably due to a partial inhibition of the enzyme by heat. In order to test the validity of this assumption, the influence of heat on the activity of reductase has been investigated. The results obtained, which permit the determination of the "destruction temperature" of reductase, and the influence of light and radium radiations on the activity of reductase are dealt with in the present communication.

Changes produced by radiations in the activity of reductase were determined by comparing the time required for the reduction of oxyhemoglobin by a quantity of the enzyme which had been acted upon by a particular radiation, with the time required for the reduction by the same quantity of the enzyme which had not been acted upon by the radiation. The progress

[2] J. R. Green: *Phil. Tr. Roy. Soc.*, clxxxviii, p. 167, 1897.
[3] V. Henri and A. Mayer: *Compt. rend. Soc. de biol.*, lvi, p. 230, 1904.
[4] S. Loewenthal and J. Wohlgemuth: *Biochem. Ztschr.*, xxi, p. 476, 1909.
[5] P. F. Richter and H. Gerhartz: *Berl. klin. Wchnschr.*, xlv, p. 646, 1908.
[6] D. F. Harris and H. J. M. Creighton: this *Journal*, xx, p. 179, 1915.

of the reduction was followed by the spectroscopic method previously described.[7] All reductions were carried out at 40°. The reductase contained in cat's fresh liver juice was employed in the experiments.

II. EXPERIMENTAL.

A. The influence of heat and temperature on the activity of reductase.

In order to determine the influence of heat and temperature on the activity of reductase, experiments were carried out as follows: A mixture of one part of liver juice and one part of water was heated for three minutes at a given temperature; the mixture was then quickly cooled to room temperature and mixed with two parts of blood solution, prepared by diluting 10 cc. of cat's freshly defibrinated blood to 250 cc. with water. This mixture was then introduced into conical observation tubes which were placed in the thermostat at 40°. The times required for the reduction of these mixtures were compared with that required for the reduction of a *normal* mixture consisting of one part of liver juice (unheated), one part of water, and two parts of the blood solution. The results of the experiments are given in Table I.

TABLE I.

4.5 minutes required for reduction of normal mixture.

TEMPERATURE AT WHICH MIXTURE OF LIVER JUICE AND WATER WAS HEATED BEFORE MIXING WITH THE BLOOD SOLUTION	TIME REQUIRED FOR THE REDUCTION OF THE MIXTURE	ACTIVITY OF THE LIVER JUICE AFTER HEATING, EXPRESSED IN TERMS OF ITS ORIGINAL ACTIVITY
	min.	*per cent*
30°	5	90
35°	6.5	69
40°	6.5	69
45°	8	56
50°	10	45
55°	11	41
60°	12.5	36
65°	14	32
70°	46	9.8
75° 80° 90°	No reduction at the end of two hours	ca.0

[7] Harris and Creighton: *loc. cit.*

Examination of Table I shows that even when the juice is heated for three minutes at as low a temperature as 30°, there occurs an appreciable decrease in its activity. As the temperature of the heating of the juice is increased, the activity of the juice shows a corresponding decrease, *i.e.*, an increase in the time required to effect reduction. This increase in the time required for re-

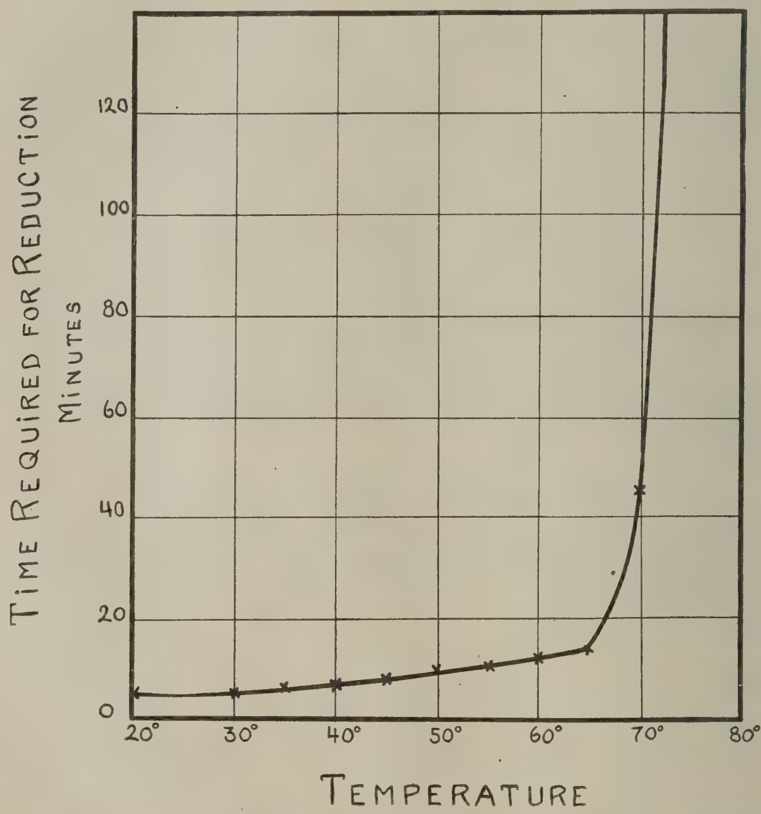


Fig. 1.

duction is gradual until the temperature reaches 65°; above this temperature it lengthens very rapidly. These results are expressed graphically in Figure 1. In this figure the temperatures at which the liver juice was heated are plotted on the axis of abscissae

against the times required for reduction on the axis of ordinates. It will be seen that between 20° and 65° the slope of the curve is quite gradual, but that at the latter temperature it suddenly becomes very steep, almost vertical. In other words, between 65° and 70° the activity of the reductase in liver juice is rapidly destroyed. When the juice is heated for three minutes at 75°, it is incapable of effecting the reduction of oxyhemoglobin at 40° in two hours; *i.e.*, juice which has been heated at this temperature behaves like a boiled control. It must be inferred, therefore, that a temperature of over 70° is sufficient to bring about complete, permanent inhibition of the enzyme. We define the destruction temperature of reductase as follows: *The destruction temperature is that temperature above which it is impossible to heat the enzyme for a given time without completely destroying its reducing power.* The destruction temperature of reductase, with three minutes' heating, therefore, is about 72°.

B. The influence of radium and light radiations on the activity of reductase.

The influence of light and radium radiations on the activity of reductase was determined by comparing the times required for the reduction of oxyhemoglobin by liver juice which had been subjected to the radiation, with that required by liver juice which had been kept in the dark. For the radium experiments 5 mg. of the bromide of an activity of about 1,000,000 were employed. The end of the small tube containing the radium was placed just above the surface of a few cc. of the liver juice. Cat's fresh liver juice was divided into three parts and placed in three narrow bottles. One of these was placed in the light, another in the dark, and the third was placed in the dark with the radium. At the end of 7, 24, 48, and 72 hours the reducing power of equal portions of each of these liver juices was measured at 40° against blood. In all the measurements, no difference was found between the times required for the reduction of the oxyhemoglobin by the different juices.

No satisfactory explanation can be put forward to account for the fact that neither light nor radium seems to exert any inhibitory action on reductase. It might have been thought that

since reductase must do its work in the depths of the tissues, that is, in complete darkness, light would have had some injurious influence upon it. This does not seem to be the case. As regards radium, it is known that its general tendency is to destroy living tissues. It would not have been surprising had it been found that radium destroyed reductase; no evidence has been obtained that it does.

III. SUMMARY.

1. The influence of heat, light, and radium radiations on the activity of reductase has been investigated.

2. It has been found that heat inhibits the activity of reductase, the extent of the inhibition depending upon the temperature. The degree of inhibition increases slowly with temperature, complete permanent inhibition taking place between 70° and 75°.

3. The destruction temperature of reductase, which has been defined, is about 72°.

4. Light and radium radiations have no appreciable influence on the activity of reductase.

FURTHER STUDIES ON THE RELATION OF DIET TO TRANSMISSIBLE TUMORS.

By J. E. SWEET, ELLEN P. CORSON-WHITE, and G. J. SAXON.

(*From the Laboratory of the American Oncologic Hospital, Philadelphia.*)

(Received for publication, April 8, 1915.)

In the first report of our work upon the possible relation of the constituents of the diet to the growth of the transmissible tumors of rats and mice[1] we described experiments carried out upon rats and mice which were fed a modification of one of the growth-inhibiting diets used by Mendel and Osborne in their studies of growth.[2] Our work has been continued, and we wish to report the results of later experiences.

The work has been continued chiefly with the use of the white rat and the Flexner-Jobling carcinoma. The observations mentioned in our earlier report in regard to the use of the litter unit have been extended and confirmed; namely, that the susceptibility or immunity toward a tumor shown by an individual is a property common to all the individuals of that litter. Therefore the most crucial experiment possible is to inoculate a large number of litters, choose those litters in which a positive tumor take is recorded, and then divide these litters equally among control and experiment.

Through the kindness of the Wistar Institute we have been able to obtain the animals in the original litters, and we feel confident of the importance of the suggestion made previously, that observations of litter units are of more value in tumor work than studies of large numbers of mixed animals.

The results of our earlier work, that animals kept upon a diet which inhibits the normal growth of the animal are hereby placed

[1] J. E. Sweet, E. P. Corson-White, and G. J. Saxon: this *Journal*, xv, p. 181, 1913.

[2] T. B. Osborne and L. B. Mendel: *Ztschr. f. physiol. Chem.*, lxxx, p. 307, 1912.

under conditions in which, after inoculation with a transmissible tumor, a diminished number of takes and an increased number of retrogressions are observed, have been fully confirmed, as shown in the following table.

TABLE I.

LITTERS	RATS	TAKES	RETRO-GRESSIONS	METAS-TASES	DIET
19.	134	72=54%	3	0	Normal
11.	82	14=17%	8	0	Mendel-Osborne

Applying our standard of a crucial experiment, inoculating many litters, and dividing those litters in which a positive take is recorded, we find the results shown in Table II.

TABLE II.

	RATS	TAKES	RETRO-GRESSIONS	METAS-TASES	DIET	DIAMETER OF TUMOR AT 30 DAYS
						mm.
12 litters of 86 rats.	43	41	7	0	Normal	10–25
	43	43	20	0	Mendel-Osborne	4–16

Since the appearance of our first paper, Robertson and Burnett have published experiments showing that cholesterol accelerates the growth of carcinoma in rats, and also greatly increases the extent of metastatic growth.[3] It seemed to us of interest to repeat these experiments and also to determine the effect of cholesterol on animals living upon the modified Mendel-Obsorne diet. The results of these experiments are shown in the following tables.

TABLE III.

LITTERS	RATS	TAKES	RETRO-GRESSIONS	METAS-TASES	DIET
11.	84	56	3	0	Normal.
9.	52	37	1	31	Normal + cholesterol on tumor.
5.	34	25	2	19	Normal + cholesterol 0.2 gm. injected on other side.
4.	26	26	0	26	Normal + cholesterol 0.2 gm. injected once a week.
5.	28	28	0	28	Normal + cholesterol 0.2 gm. fed once a week.

[3] T. B. Robertson and T. C. Burnett: *Jour. Exper. Med.*, xvii, p.344, 1913.

TABLE IV.

LITTERS	RATS	TAKES	RETRO-GRESSIONS	METAS-TASES	DIET
11	84	56	3	0	Normal.
23	140	116	3	104	Normal + cholesterol.

TABLE V.

LITTERS	RATS	TAKES	RETRO-GRESSIONS	METAS-TASES	DIET
11......	54	9	2	0	Mendel and Osborne.
3......	20	5	2	2?	Mendel and Osborne + cholesterol on tumor.
2......	14	2	1	0	Mendel and Osborne + cholesterol injected on other side.
2......	11	5	0	1?	Mendel and Osborne + cholesterol injected 0.2 gm. once a week.
2......	14	4	0	0	Mendel and Osborne + cholesterol fed 0.2 gm. once a week.

Mendel and Osborne diet for two weeks before inoculation.

TABLE VI.

LITTERS	RATS	TAKES	RETRO-GRESSIONS	METAS-TASES	DIET
11.......	54	9	2	0	Mendel and Osborne.
9.......	59	16	3	3?	Mendel and Osborne + cholesterol.

Applying again our standard of the crucial experiment, the inoculation of a large number of litters, and equally dividing those in which a positive take is recorded, we obtain the result shown in Table VII.

TABLE VII.

	RATS	TAKES	RETRO-GRESSIONS	METAS-TASES	DIET
9 litters of 111 rats..........	52	42	4	34	Normal + cholesterol.
	59	16	3	3?	Mendel and Osborne + cholesterol.

It might be more or less pertinently suggested that such an experiment, to be entirely above criticism, should be made upon a series of animals all inoculated from the same original tumor. Such an experiment is found in Table VIII.

TABLE VIII.

Mar. 6, 1913. Nine litters were inoculated with the same tumor, a very large, rapidly growing one; three litters showed a positive take in every individual. These litters were divided as follows:

1 litter of 12 rats: 6 on normal diet + cholesterol; 6 on Mendel-Osborne diet + cholesterol.

1 litter of 6 rats: 3 on normal diet + cholesterol; 3 on Mendel and Osborne diet + cholesterol.

1 litter of 7 rats: 3 on normal diet + cholesterol; 4 on Mendel and Osborne diet + cholesterol.

RATS	TAKES	RETRO-GRESSIONS	METAS-TASES	DIET
12	12	0	12	Normal + cholesterol.
13	13	0	1 (lung)	Mendel and Osborne + cholesterol.

In the above tables the question mark placed after the figures representing the metastases found in the cholesterol-treated animals on the Mendel and Osborne diet, means that these metastases were simply enlarged glands or secondary tumor growths in the immediate neighborhood of the primary inoculation, whereas the metastases in the cholesterol animals on the normal diet were all into distant organs, such as the lungs, mediastinal glands, liver, etc. In only one cholesterol animal, maintained on the Mendel and Osborne diet, did we find a metastasis into a distant organ, in this case into the lung.

It is a much simpler matter to obtain results such as those contained in the preceding tables, than it is to present a fair and critical interpretation of them. Our work has been based entirely upon the work instigated by Mendel and Osborne and substantiated by numerous other observers, and the viewpoint has been expressed by them in the following words:[4]

Our own experiments, as well as those of the other investigators mentioned, make it clear that something further than a sufficient supply of

[4] Osborne and Mendel: this *Journal*, xv, p. 312, 1913.

energy-yielding food material is required to promote a normal growth. The animal cells need for their activities not only energy, but also suitable constructive material to replace the wear and tear therein. Furthermore the cells are concerned in the elaboration of a great diversity of complex and little understood substances such as enzymes, products of internal secretion, etc., which unquestionably play an indispensable rôle in life and may require either special antecedent products for their construction, chemical activators of some sort, or minute quantities of readily overlooked rarer elements and compounds. It is easy, yet futile at the present time, to develop detailed hypotheses respecting the almost innumerable possibilities involved.

One of the most concrete examples of this concept of substances essential for normal cell reproduction and growth, which substances have nothing to do with general malnutrition, is seen in the effect upon spermatogenesis of the removal of the pituitary. Such an animal may double its original weight, but the spermatogenetic tissue of the testicle completely disappears. It is supposed that a hormone is formed by the pituitary which controls spermatogenesis; but this hormone is a chemical body and must be built up by the pituitary from chemical compounds which have been furnished by the rest of the body or some portion of the body. These components of the finished hormone must have been derived in the last analysis from the food or possibly from the breakdown of the tissues of the body itself. Therefore the lack of such essential, though unknown components of the food,—provided that the body is not breaking down at a rate sufficiently rapid to supply these components,—should result in a lack of specific substances.

It is of interest in this connection to note that substances which are inhibitory of tumor growth are also destructive of the normal process of spermatogenesis. The X-ray, radium, and injection of choline all inhibit or destroy tumor activity, and all likewise are known to destroy spermatogenesis;[5] certain of our rats on the Mendel-Osborne diet have also shown a complete loss of spermatogenesis.

Since the appearance of our first paper, several series of experiments have been reported along similar lines. Van Alstyne and Beebe[6] found that a diet of casein and lard exerted a depressing

[5] R. Werner and St. Szécsi: *Ztschr. f. Chemotherap.*, i, p. 357. 1912–13.

[6] E. V. N. Van Alstyne and S. P. Beebe: *Jour. Med. Research*, xxiv, p. 217, 1913–14.

effect upon tumor growth, as compared with the growth of animals fed upon a diet of bread.

They found also that twenty-four out of twenty-six animals on a diet of casein, lard, and lactose grew a tumor with fatal results, while on a diet of casein and lard, twenty regressed and only two were killed by the tumor. They conclude that the carbohydrate element in the diet is the one responsible for the difference.

Two facts may be pointed out in this work of Van Alstyne and Beebe. First, the fact that a diet of casein and lard does not contain any of these unknown substances which are essential for normal growth, whereas lactose has in our own experience sometimes apparently contained these substances. We have found it impossible to maintain rats at a constant level of weight on certain preparations of lactose. The other point is in regard to the actual condition of the animals reported in these series. For instance, the average weights of two series of animals are given[7] during the first four weeks after inoculation with the tumor.

It will be seen from these tables that upon a special diet rats weighing 41.7 grams reached a weight of 48 grams after four weeks; and on the control diet, rats weighing 42.3 grams averaged 45.1 grams four weeks later; according to Donaldson's table of the normal growth of the white rat,[8] an animal weighing 42 grams should weigh 106 grams four weeks later. Or[9] a series averaging 72.1 grams on the special diet, weighed 78.6 grams four weeks later, and the control series on the regular diet weighing 60.4 grams, averaged 69.2 grams four weeks later. According to the same authority on the normal rat, rats weighing about 72 grams should weigh 148 grams four weeks later, and rats weighing about 60 grams should weigh 135 grams four weeks later.

That the animals had an abundance of food and appeared to be in excellent nutritional condition is entirely in accord with the proper results of the Mendel and Osborne data, and it would seem to us that the studies reported by Van Alstyne and Beebe might be considered almost as comparative results of two diets, each of which inhibits normal growth; nor does such a statement necessarily disagree with our previous conclusion that tumor

[7] Van Alstyne and Beebe: *loc. cit.*, p. 226.
[8] H. H. Donaldson: *Boas Anniversary Volume*, New York, 1906, p. 5.
[9] Van Alstyne and Beebe: *loc. cit.*, p. 223.

cells agree with normal somatic cells with respect to their laws of growth. If we could stop all normal cell growth we could doubtless stop tumor cell growth, but where any cell growth is possible, we believe that experimental work and clinical findings both indicate that the tumor cell retains its power of growth longer than the somatic cell, and it is in this respect of possessing a stronger affinity for the growth-producing substance that the tumor cell differs from the normal cell.

Another interesting article concerns us here—the work of Rous on "The influence of diet on transplantable and spontaneous mouse tumors."[10] The study of Rous's paper leads us to the conclusion that his work substantiates our earlier findings and that the only differences of note are differences in the point of view. The influence of underfeeding must undoubtedly be taken into consideration in the study of tumor growth, and must also be ruled out in such studies as we have undertaken upon the effects of diet. In all of our experiments animals which lost in weight on the special diet were not considered fair objects of experiment and were rejected, unless it were that animals which developed a tumor, even on a normal diet, showed a gradual decline in weight after a long period.

A study of the following weight curves, which have been compiled as average curves of our experimental animals, might lead one to conclude with Rous that the element of underfeeding enters largely into the results. It will be noticed that the animals kept on a normal diet plus cholesterol show an average weight curve more nearly approximating the normal, and that the lowest average curve is that represented by the inoculated animals on a gliadin diet; but a study of the individual weight curves of some of these animals shows that some factor other than malnutrition must have entered.

Thus a rat weighing 56 grams at the time of inoculation, February 27, 1914, reached a maximum weight of 68 grams on May 4, and weighed only 42 grams at the time of death, July 4. The animal died with a small tumor and many metastases. An animal weighing 53.8 grams. inoculated on March 16, 1914, reached a maximum weight of 112 grams on May 28, and died weighing

[10] P. Rous: *Jour. Exper. Med.*, xx, p. 433, 1914.

99.8 grams on July 5, with a tumor weighing 22.4 grams and with many metastases. According to Donaldson's standard table, a rat weighing 56 grams should have weighed four months later about 230 grams, and a rat weighing 53.8 grams should also have weighed about 230 grams, approximately four months later.

At the same time another animal weighing 96.3 grams at the time of inoculation, December 20, 1913, reached a maximum weight of 259.2 grams on May 10, and died on June 6 weighing

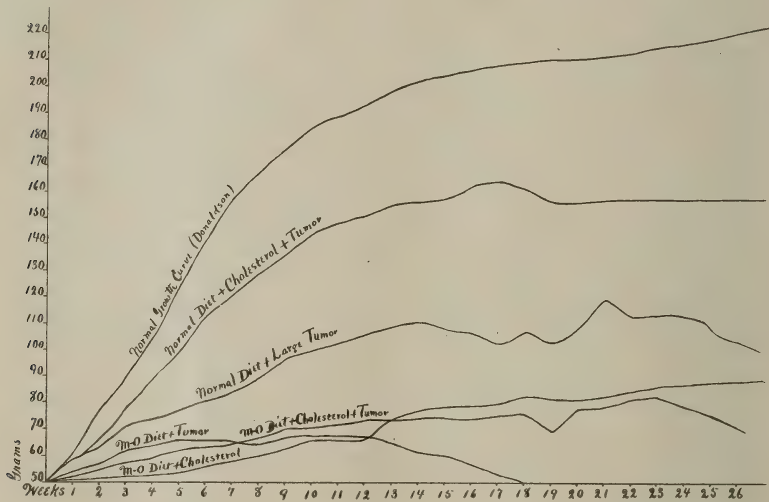


FIG. 1.

207.4 grams, with a tumor weighing 15.3 grams and many metastases. According to Donaldson's table such an animal should have weighed about 260 grams at the time of its death, and in fact the animal did reach a maximum weight of 259.2 grams.

A comparison of the average weight curves shown in the table would at first sight seem to indicate, as we have said, that the question of tumor growth was entirely parallel with the normal growth of the organism. The animals which showed the most extensive growth of tumor and development of metastases, those on a normal diet plus cholesterol, show a growth curve which for a long period closely approximates the normal. The animals on a normal diet with a tumor fall far below this curve.

The next best curve is that on the gliadin diet plus cholesterol, and the lowest curve is represented by those animals on the gliadin diet possessing a tumor. These curves illustrate a possible difference in point of view, such as we think constitutes the chief difference between the work of Rous and our early results; but the study of individuals in our series, which in spite of the normal diet and the addition of cholesterol show every evidence of stunted general growth and nevertheless grew large tumors with many metastases, can only be interpreted as meaning that some factor of diet permitted the growth of the tumor, even though the animal itself failed to grow normally. The results obtained with the use of cholesterol are also interpreted by us as showing the effect of substances not comprised under the general head of proteins, carbohydrates, or fats, upon the growth of tumors, as shown in Table IV. Out of 116 animals, 104 developed metastases on the addition of cholesterol, and we have never had a metastasis with this tumor in our laboratory under any conditions except on the addition of cholesterol. We are nevertheless unable to agree with Robertson and Burnett that cholesterol is a factor of decisive importance. If it were cholesterol alone which is responsible for the increased tumor growth, it is hardly conceivable that the results shown in Table VIII could be obtained.

A study of Tables III and V shows that animals placed on the Osborne and Mendel diet have developed, in spite of the cholesterol, the same resistance toward tumor takes that has characterized our work with this diet. It would seem, therefore, that the action of the cholesterol must depend not upon its action alone, but upon its action when combined with some unknown element of the food. The effect of cholesterol added to a normal diet in producing metastases of a tumor which had never before in our hands shown a metastasis is certainly remarkable, but the fact that this same substance, when used in the presence of a growth-inhibiting diet, has only in one instance produced a development of a metastasis into a distant organ, is equally remarkable.

We believe that our further experience, as outlined above, supports our earlier conclusion that the tumor cells are subject to the same laws of growth as the normal somatic cells, the only difference being that the tumor cell possesses a stronger avidity

for the unknown substances in the diet which are essential for cell growth, and will therefore persist in its development even though the individual is losing weight. It must also be borne in mind that this unknown substance might also be obtained from the breakdown of normal body cells and that experiments along such lines can only be considered satisfactory when the individuals are kept under conditions in which there is no loss of weight.

RESEARCHES ON PURINES. XVII.[1]

ON A NEW SYNTHESIS OF ALKYLAMINO-PURINES. ON 2-OXY-8-THIOPURINE, 2-OXY-8-METHYLMERCAPTO-PURINE, 2-OXY-8-METHYLAMINO-PURINE AND 2-OXY-6, 9-DIMETHYL-8-THIOPURINE.

By CARL O. JOHNS.

(*From the Sheffield Laboratory of Yale University, New Haven.*)

(Received for publication, April 12, 1915.)

Very few alkylamino-purines are known. Cramer,[2] working in Emil Fischer's laboratory, prepared 8-methylaminocaffeine (II) and 8-ethylaminocaffeine (III) by heating 8-chlorcaffeine (I) with methyl- and ethylamine, respectively. This method is limited in its application because the chlor-derivatives of many simple purines have not been prepared. Although uric acid gives chlor-derivatives, the simple mono- and di-oxy-purines do not seem to react with the phosphorus halides to give the corresponding chlor-derivatives. This is probably because they are more basic in nature than is uric acid.

A considerable number of purine derivatives containing sulphur have been prepared. When such purines are alkylated with methyl iodide, the methyl group becomes attached to the sulphur atom,[3] and a methylmercapto-purine is obtained. It is probable that this methylation could be accomplished by means of dimethyl sulphate instead of methyl iodide, since dimethyl sulphate has been used successfully in methylating on the sulphur atom in pyrimidines.[4]

The writer has found that methylamine reacts smoothly with methylmercapto-purines to form methylamino-purines, methyl-

[1] C. O. Johns and B. M. Hendrix: this *Journal*, xx, p. 153, 1915. The present investigation was aided by a grant from the Bache fund.

[2] L. Cramer: *Ber. d. deutsch. chem. Gesellsch.*, xxvii, p. 3089, 1894.

[3] E. Fischer: *ibid.*, xxxi, p. 431, 1898.

[4] C. O. Johns and E. J. Baumann: this *Journal*, xiv, p. 384, 1913.

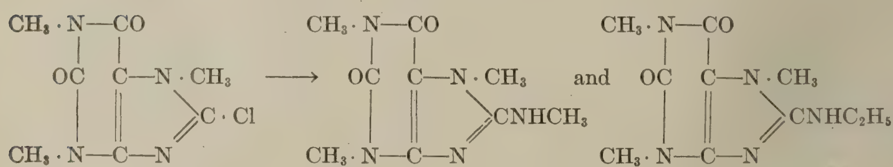
mercaptan being liberated. It is probable that the higher alkyl amines as well as the aryl amines will also react with these mercapto-purines, thus giving a variety of new alkyl and aryl amino-purines.

A preliminary experiment also indicated that ammonia at a high temperature reacts with 2-oxy-8-methylmercapto-purine (VI) and liberates methylmercaptan. The resulting 2-oxy-8-amino-purine was not obtained in a pure state, and hence a description of this compound must be deferred until more experimental work can be done.

When 2-oxy-5,6-diamino-pyrimidine (IV)[5] was heated with thiourea or ammonium thiocyanate at 185° C., 2-oxy-8-thiopurine (V) was obtained. When the sodium salt of 2-oxy-8-thiopurine was suspended in alcohol containing methyl iodide, and this mixture was warmed, alkylation took place on the sulphur atom and 2-oxy-8-methylmercapto-purine (VI) was formed. This compound reacted readily with methylamine at 100°C. and gave 2-oxy-8-methylamino-purine (IX).

This communication also contains a description of the method of preparation and the properties of 2-oxy-6,9-dimethyl-8-thiopurine (VIII). This compound was prepared by heating 2-oxy-4-methyl-5-amino-6-methylamino-pyrimidine (VII)[6] with thiourea.

These researches will be continued.



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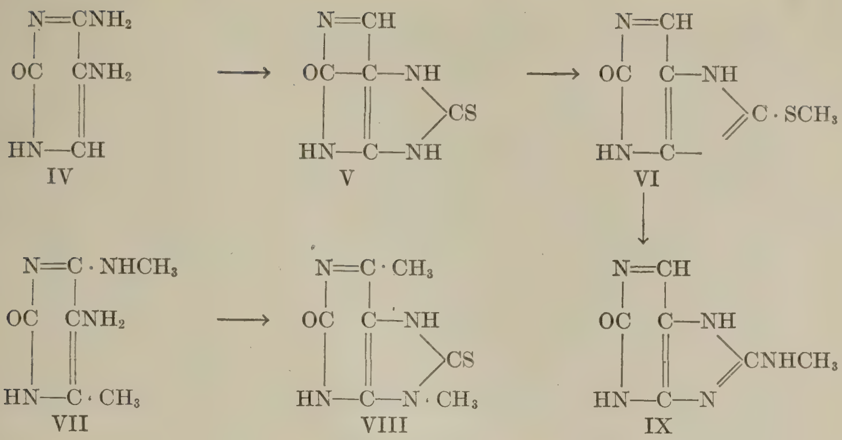
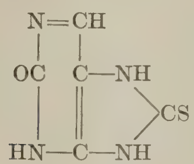
[5] Johns: *Am. Chem. Jour.*, xlv, p. 82, 1911.
[6] Johns and Baumann: this *Journal*, xv, p. 123, 1913.



EXPERIMENTAL PART.

2-Oxy-8-thiopurine.

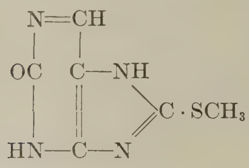


One gram of 2-oxy-5,6-diamino-pyrimidine[7] was heated together with 1 gram of thiourea in an oil bath at 180° to 185° C. for an hour. The mixture melted and finally formed a hard, black crust. This was boiled in dilute ammonia and the insoluble portion was filtered off and discarded. The filtrate was decolorized with blood coal and then acidified with acetic acid. This treatment produced a precipitate of very minute prisms which united to form globular aggregations. These crystals were difficultly soluble in hot water and in the common organic solvents. They were purified by dissolving in dilute ammonia and reprecipitating with acetic acid. They did not melt at 300° C.

	Calculated for $C_5H_4ON_4S$:	Found.
N	33.33	33.31
S	19.04	18.28

[7] Johns: *loc. cit.*

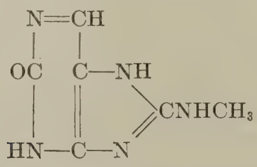
2-Oxy-8-methylmercapto-purine.



The sodium salt of 2-oxy-8-thiopurine was prepared by dissolving the purine in a solution of sodium ethylate and water. 0.3 of a gram of metallic sodium was dissolved in a little alcohol and 10 cc. of water were added. Two grams of 2-oxy-8-thiopurine were then dissolved in this solution, after which 90 cc. of alcohol were added. The sodium salt of the purine was precipitated partly by the alcohol. Four cc. of methyl iodide were added and the mixture was heated gently under a reflux condenser by means of a water bath. Alkylation took place rapidly, and after fifteen minutes the solution had ceased to react alkaline. The mixture was then evaporated to dryness and the residue dissolved in a little hot, dilute ammonia. On acidifying the hot solution with acetic acid, a precipitate of slender prisms was obtained. These were difficultly soluble in organic solvents and in water. They decomposed slowly when heated above 260° C., but did not melt at 300° C. The yield was about 60 per cent of the calculated quantity.

	Calculated for $C_6H_6ON_4S$:	Found: I	Found: II
N	30.76	30.67	30.33

2-Oxy-8-methylamino-purine.

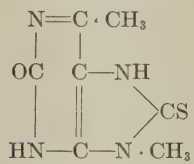


One gram of 2-oxy-8-methylmercapto-purine was dissolved in a mixture of 4 cc. of aqueous methylamine and 6 cc. of water. This solution was heated in a sealed tube at 100° C. over night. When the tube was opened there was a strong odor of mercaptan, and the original red color of the solution had disappeared. The

mercaptan was driven off by evaporating the solution to dryness. The residue did not give a test for sulphur. It dissolved but very slightly in water or the common organic solvents. It did not dissolve in dilute ammonia, but dissolved readily in dilute sodium hydroxide, and on acidifying this hot solution with acetic acid, a crystalline precipitate was obtained. The crystals were very minute and formed sheaf-like bundles. They did not melt at 300° C. The yield was about 75 per cent of the calculated quantity.

	Calculated for $C_6H_7ON_5S$:	Found:
N..	42.42	42.13

2-Oxy-6,9-dimethyl-8-thiopurine.



1.6 grams of 2-oxy-4-methyl-5-amino-6-methylamino-pyrimidine[8] were mixed with 2 grams of thiourea by grinding the substances together in a mortar. This mixture was heated in an oil bath at 170° to 180° C. for an hour. The reaction-product was then dissolved in hot, dilute ammonia, and the solution was decolorized with blood coal. The solution was filtered while still hot, and the filtrate was acidified with acetic acid, whereupon a precipitate was obtained. This was purified by dissolving again in hot, dilute ammonia and reprecipitating with acetic acid. In this way the purine was obtained as a light colored, finely divided powder. The yield was almost quantitative. This purine was but slightly soluble in hot alcohol or water and did not dissolve in benzene. When evaporated with nitric acid on the steam bath it gave a yellow residue which turned red when moistened with ammonia. The red color was intensified by heating gently.

	Calculated for $C_7H_8ON_4S$:	Found:
N..	28.57	28.67

[8] Johns and Baumann: *loc. cit.*

VIVIDIFFUSION EXPERIMENTS ON THE AMMONIA OF THE CIRCULATING BLOOD.

By ALICE ROHDE.

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(Received for publication, April 25, 1915.)

The fact that the ammonia content of shed blood under aseptic conditions may increase or diminish over a period of observation of twenty-four hours was reported by Medwedew.[1]

The amount of ammonia was found to increase as much as 1.4 mg. per 100 cc. in twenty-four hours in blood from normal, well nourished dogs and to fall as much as 0.8 mg. per 100 cc. in twenty-four hours in blood from animals which had fasted for forty-five days. Medwedew explains the production of the ammonia by a deamidization process due to ferment action upon substances in the blood, which may yield ammonia and the disappearance of ammonia by a synthetic process due also to ferment action in which the ammonia of the blood enters into the formation of an amino or amido compound. Both ferments Medwedew assumes to be present in the plasma. His experiments lead him also to believe that a deamidase diffuses slowly from the corpuscles in shed blood. Folin[2] emphasizes the process of ammonia formation in these words: "The blood decomposes spontaneously at all temperatures even when kept on ice. The ammonia thus produced by decomposition in the course of a few hours is much greater than the preformed ammonia present in the strictly fresh blood." Grafe[3] speaks of labile substances in the tissues of recently killed animals which easily split off ammonia.

The following experiments were undertaken to determine whether with aseptic measures the formation of ammonia occurs in diffusible constituents of the blood after their separation from the non-diffusible constituents according to the method of Abel, Rowntree, and Turner.[4] A vividiffusion apparatus was attached

[1] A. Medwedew: *Ztschr. f. physiol. Chem.*, lxxii, p. 410, 1911.

[2] O. Folin and W. Denis: this *Journal*, xi, p. 532, 1912.

[3] E. Grafe: *Ztschr. f. physiol. Chem.*, xlviii, p. 302, 1906.

[4] J. J. Abel, L. G. Rowntree, and B. B. Turner: *Jour. Pharm. and Exper. Therap.*, v, p. 275, 1913–14.

to the circulation of well nourished dogs and the dialysate studied for the production of ammonia in excess of that present at the time of dialysis. The results were compared with those obtained with shed blood under similar conditions.

The Folin[5] method for the determination of ammonia in blood was used for the analyses. 100 cc. of the material were measured into a 1000 cc. cylinder, and 10 cc. of 0.01 normal hydrochloric acid were measured into each of two 200 cc. Erlenmeyer flasks and diluted with about 10 cc. of water. A duplicate sample and a sample of a standard solution of ammonium sulphate containing 0.28 mg. of ammonia nitrogen per 100 cc. were arranged in a similar apparatus, and one pump attached directly to the water-main was used at full force to draw an air current through this train of cylinders, receivers, and wash bottle. 20 cc. of a saturated solution of sodium carbonate were used to free the ammonia from each sample of material. Small bubblers made in the laboratory after the Folin pattern were used in the absorption apparatus. Water redistilled from phosphoric acid was used in diluting and in rinsing. The samples were aerated for three hours, although two hours usually were found sufficient for the standard solution and for the dialysate. To prevent foaming 25 cc. of ethyl alcohol and 10 cc. of toluene were added to each sample and renewed when necessary. 0.01 normal sodium hydroxide and one drop of 1 per cent sodium alizarinsulphonate were used in titrating the excess of acid. In 0.3 mg. of ammonia nitrogen per 100 cc. of material there is a probable error of 4 per cent in the procedure as here carried out, but as the results in these experiments are of value because of their comparative interest rather than the absolute ammonia content of the material, it was thought unnecessary to attempt to reduce the error below this value. In Experiment V use was made of Folin's[6] microchemical method for the determination of ammonia nitrogen in the blood in order to determine quickly during a process of diffusion the ammonia content of a dialysate and the ammonia content of the blood with which it might have reached an equilibrium.

A vividiffusion apparatus designed to hold 1600 cc. of dialysate and 200 cc. of blood according to the model represented in Figure 1 was used for Experiments I to IV. An apparatus represented by Figure 2 was used for Experiment V. This latter apparatus contained only 150 cc. of dialysate and 100 cc. of blood. Precautions were taken in all experiments to secure good mixing of the outer fluid. In the first experiment a rubber bulb filled with sterile saline was attached to the inflow and to the outflow tubes of the outer jacket and pressed every eight seconds by hand. In the second experiment a centrifugal pump was connected by outflow and inflow tubes to the outer jacket of the apparatus and a rapid and uniform circulation

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[5] O. Folin: *Ztschr. f. physiol. Chem.*, xxxvii, p. 161, 1902.

[6] Folin and Denis: *loc. cit.*, p. 528.

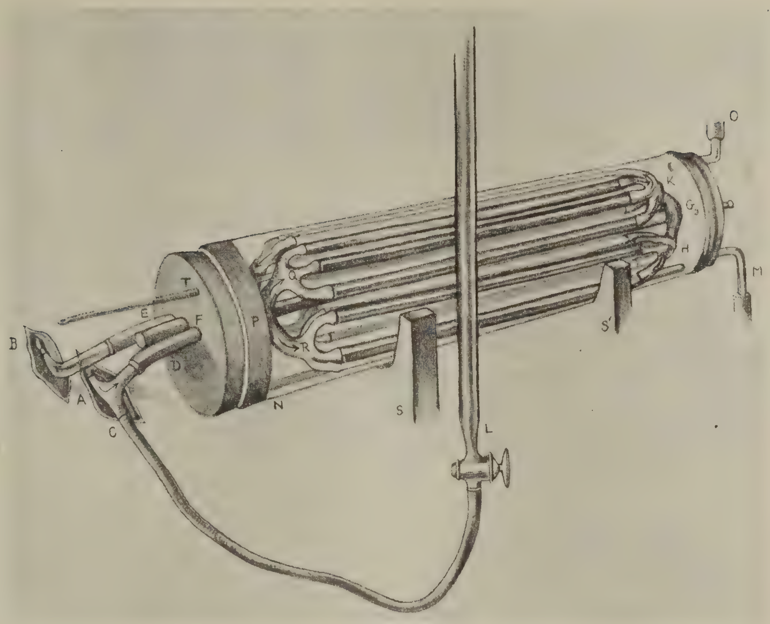

FIG. 1. PERSPECTIVE VIEW OF VIVIDIFFUSION APPARATUS.

A, arterial cannula; *B*, venous cannula; *C*, side tube for introduction of hirudin; *D*, inflow tube; *E*, outlet tube; *F*, *G*, supporting rod attached at *H* and *K* to branched U-tubes; *L*, burette for hirudin; *M*, *N*, tube for filling and emptying liquid in outer jacket; *O*, air outlet; *P*, dichotomous branching point of inflow tube; *Q* and *R*, quadruple branching points of same; *S*, *S′*, wooden supports; *T*, thermometer. At each of the points *H* and *K* the blood is collected from four tubes into one, bending around to the back, and there redividing into four return flow tubes. Arrows show the direction of flow.

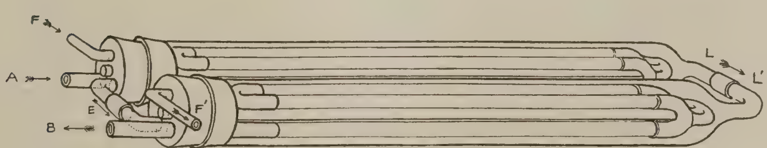

FIG. 2. *A*, blood inlet; *B*, outlet; *F*, inlet for saline solution; *F′*, outlet for same. The connections for the blood stream by the tube *E* and for saline solution at *L*, *L′* are made by rubber tubing.

of the dialysate obtained. When not in use the vividiffusion apparatus was kept filled with a saturated solution of thymol in distilled water to preserve as nearly as possible aseptic conditions in the celloidin tubes. Before use the apparatus was thoroughly rinsed with 0.65 per cent sterile saline at 40° and completely filled. The preparation and use of the hirudin solution were according to the directions given in the reference already cited.

Experiment I. The results show the formation of ammonia in shed blood. A specimen of dog blood was drawn with aseptic precautions into a flask containing dry sodium oxalate, and portions of it were analyzed for ammonia at once and others after standing on ice for twenty-four hours with chloroform and toluene. The aeration was carried out at room temperature. A bacteriological examination of the blood which had stood twenty-four hours was made and gave negative cultures.

TIME	NH_3 N PER 100 CC. OF BLOOD
	mg.
0 hrs. + 3 hrs. aeration	0.72
24 hrs. + 3 hrs. aeration	1.78

Experiment II. The results show the formation of ammonia in shed blood. A specimen of dog blood was drawn under aseptic conditions and treated as in the previous experiment. In addition, however, the aeration apparatus was previously sterilized and the cylinders were kept in ice during the process of aeration.

TIME	NH_3 N PER 100 CC. OF BLOOD
	mg.
0 hrs. + 3 hrs. aeration	0.28
24 hrs. + 3 hrs. aeration	0.44

Experiment III. The results show that no ammonia is formed from the diffusible constituents of the blood. A well nourished dog weighing 15 kilos was anesthetized with ether and the vividiffusion apparatus attached under aseptic precautions to the right femoral artery and to the left femoral vein. 130 cc. of hirudin solution were allowed to mix slowly with the blood through the arterial cannula. Dialysis was carried on for three hours with stirring of the dialysate. Duplicate portions of the dialysate were immediately aerated. The remainder of the material

was preserved with chloroform and toluene in sterilized flasks on ice until aerated.

TIME	NH_3 N PER 100 CC. OF DIALYSATE
	mg.
0 hrs. + 3 hrs. aeration	0.17
24 hrs. + 3 hrs. aeration	0.18

Experiment IV. The results show that no ammonia is formed from the diffusible constituents of the blood. A well nourished dog weighing 12 kilos was given intraperitoneally 3.6 grams of chloretone ground up in olive oil, and ether was administered while the left femoral vein and right femoral artery were exposed under aseptic precautions and fitted with the venous and the arterial cannulae. 100 cc. of hirudin solution were used. Dialysis took place for three hours with stirring of the outer fluid.

TIME	NH_3 N PER 100 CC. OF DIALYSATE
	mg.
0 hrs. + 3 hrs. aeration	0.18
64 hrs. + 3 hrs. aeration	0.18
107 hrs. + 3 hrs. aeration	0.18

Experiment V. The results show: (1) an equilibrium between the ammonia of the circulating blood and dialysate; and (2) the formation of ammonia in shed blood on standing, as compared to the absence of this formation in the dialysate. A well nourished dog weighing 14 kilos was given 3.5 grams of chloretone by stomach tube one hour before the operation. The apparatus was attached to the right femoral artery and to the left femoral vein under aseptic precautions. 200 cc. of hirudin solution were given. Dialysis took place for seven hours.

Determinations on 10 cc. of material by the microchemical method.

TIME OF DIALYSIS	DIALYSATE NH_3 N PER 100 CC. OF MATERIAL
hrs.	*mg.*
$2\frac{1}{2}$	0.33
$5\frac{1}{2}$	0.27
7	0.29
At close of experiment	BLOOD 0.30 mg.

Determinations on 50 cc. of material by the macro method.

TIME AFTER DIALYSIS	DIALYSATE NH_3 N PER 100 CC. OF MATERIAL
36 hrs. + 3 hrs. aeration	0.28 mg.
	BLOOD NH_3 N PER 100 CC. OF MATERIAL
36 hrs. + 3 hrs. aeration	0.63 mg.

SUMMARY.

It has been shown, in agreement with the findings of Medwedew, that in blood taken aseptically and kept under aseptic conditions there is a liberation of ammonia. In a dialysate obtained from circulating blood by the vividiffusion method there is no liberation of ammonia comparable to that which takes place under aseptic conditions in shed blood. The ammonia content of the dialysate when equilibrium with the blood is reached equals that of the sample of the circulating blood. The source of the slowly liberated ammonia is to be sought in the non-dialyzable constituents of the blood.

ON THE SYNTHESIS OF HIPPURIC ACID IN THE ANIMAL ORGANISM AND THE OCCURRENCE OF FREE BENZOIC ACID IN THE URINE.

By G. W. RAIZISS and H. DUBIN.

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(Received for publication, April 30, 1915.)

The elimination of hippuric acid after the administration of sodium benzoate has been the subject of many investigations, and considerable information of value has been obtained. In recent years, attention has been directed to the process of formation of hippuric acid in the body, chiefly because it was hoped that it might be possible to throw some light on certain phases of the intermediary protein catabolism.

The appearance in the urine of large amounts of glycocoll conjugated with benzoic acid suggests at least two possibilities. First, that glycocoll is the product into which most of the amino-acids are converted, prior to being excreted as urea and ammonia. Secondly, that glycocoll is produced synthetically during a special process of detoxication of benzoic acid.

The solution of these problems was attempted by many, but satisfactory proof in favor of one or the other theory is still to be established. From the literature on this subject we see that in general two methods of investigation were employed. By the first, the amount of glycocoll excreted in the urine was estimated on the basis of hippuric acid elimination. The glycocoll nitrogen thus obtained was compared with the total nitrogen, urea, and other end-products of metabolism. This procedure, for instance, was taken by Wiechowski,[1] Parker and Lusk,[2] Ringer,[3] McCollum and Hoagland,[4] Lewis,[5] and others. By the second, large amounts of various

[1] W. Wiechowski: *Beitr. z. chem. Phys. u. Path.*, vii, p. 204, 1906.

[2] W. H. Parker and G. Lusk: *Am. Jour. Physiol.*, iii, p. 472, 1900.

[3] A. I. Ringer: *ibid.*, x, p. 327, 1911–12.

[4] E. V. McCollum and D. R. Hoagland: this *Journal*, xvi, p. 321, 1913.

[5] H. B. Lewis: *ibid.*, xvii, p. 503, 1914.

amino-acids or other nitrogenous substances were introduced into the animal body, and their influence on the extent of hippuric acid synthesis was observed, determining the relationship between free and conjugated benzoic acid. This procedure was followed by Wiener,[6] Epstein and Bookman,[7] Abderhalden,[8] Kingsbury and Bell,[9] and others. It is apparent, however, that for both methods we must have data showing the relationship between free and conjugated benzoic acid. A review of the literature reveals that there are insufficient data on this point.

Some investigators, assuming that the synthesis of hippuric acid is complete, calculate the amount of glycocoll eliminated in the urine on the basis of total benzoic acid found. This assumption may, however, be erroneous, since total benzoic acid represents not only hippuric acid, but also sometimes considerable amounts of non-conjugated benzoic acid. On the other hand, we see many instances in which large amounts of free benzoic acid were reported, which seem to us too high, for the following reason. It has long been known that hippuric acid in the urine easily decomposes into benzoic acid and glycocoll, but this fact was not appreciated enough, and proper precautions for the preservation of the urine were not taken. We therefore thought it of interest to investigate to what extent free benzoic acid makes its appearance in the urine after feeding various amounts of sodium benzoate, taking all precautions to prevent the decomposition of conjugated benzoic acid.

Schmiedeberg[10] isolated from animal organs a ferment which possessed the power of splitting hippuric acid. He called this ferment "histozyme." Especially rich in this ferment are the tissues of the pig. The liver and kidneys of the dog also showed considerable ability to decompose hippuric acid.

Minkowski[11] corroborated Schmiedeberg's findings, but failed to find any splitting ferment in the organs of the rabbit. Hippuric acid introduced into the system of this animal remained unchanged. No trace of benzoic acid could be found in the blood or in the organs of the animal.

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[6] H. Wiener: *Arch. f. exper. Path. u. Pharmakol.*, xl, p. 313, 1897–98.

[7] A. A. Epstein and S. Bookman: this *Journal*, xx, p. 353, 1911–12.

[8] E. Abderhalden and H. Strauss: *Ztschr. f. physiol. Chem.*, xci, p. 81, 1914.

[9] F. B. Kingsbury and E. T. Bell: this *Journal*, xx, p. 73, 1915.

[10] O. Schmiedeberg: *Arch. f. exper. Path. u. Pharmakol.*, xiv, p. 379, 1881.

[11] O. Minkowski: *ibid.*, xvii, p. 445, 1883.

An interesting work, published in 1883 by van de Velde and Stokvis,[12] contradicted the results obtained by Schmiedeberg, and Minkowski. Several dogs, kept on a meat and bread diet, were given *per os* and subcutaneously from 2.5 to 5 gm. of hippuric acid. The animals were catheterized frequently during twenty-four hour periods, care being taken to preserve the urine. No traces of benzoic acid were found, hippuric acid being completely eliminated unchanged in the first twenty-four hours. It appears that dogs on a protein-rich food, giving rise to an acid urine, do not split hippuric acid. The results obtained with rabbits were somewhat different, since it was not always possible to secure an acid urine. Rabbits fed on vegetables secreted alkaline urine, and showed marked decomposition of hippuric acid, the amount of free benzoic acid being, in some cases, greater than hippuric acid. On a milk and gelatin diet, the urine was slightly acid, and the splitting of hippuric acid comparatively small. Benzoic acid was entirely absent in urines of starving rabbits to which hippuric acid was administered, the urines being acid. The authors reached the conclusion that the animal body apparently has no power of splitting hippuric acid. The appearance of free benzoic acid in the urine after the administration of hippuric acid, they explained was due to the activity of a certain ferment which develops its full power in an alkaline urine—as well in the bladder as outside the animal body.

Wiener[6] preserved the urine with formaldehyde, claiming that this prevented the decomposition of hippuric acid. He gave large amounts of benzoic acid *per os* to rabbits—in two cases about 1 gm. per kilo, and in three other cases as much as 1.5 gm. per kilo. The amounts of benzoic acid as hippuric acid were respectively as follows: 83.5, 83.8, 65.6, 65.6, 80.5 per cent.

Wiechowski's[1] findings regarding the extent of hippuric acid synthesis are of interest. He thinks that the decomposition of hippuric acid, both in and outside of the body, occurs only when the urine is alkaline, and is caused either by the activity of a ferment or of certain bacteria; hence care must be taken to prevent the excretion of an alkaline urine. For this purpose, he fed rabbits on oats; as a result, the urine was slightly acid. On the basis of a critical review of the experiments of others and also his own, he concludes that the synthesis of hippuric acid in the animal body is not quantitative. In Table XXI of his work, Wiechowski reports an experiment in which he gave a rabbit weighing 2360 gm., 1.78 gm. of benzoic acid subcutaneously. The animal excreted 0.300 gm. of free benzoic acid and 0.850 gm. of conjugated benzoic acid, a comparatively small dose, 0.75 gm. per kilo, resulting in the excretion of as much as 26.2 per cent of free benzoic acid. Further, the simultaneous administration of glycocoll and benzoic acid to the same animal leads to no decrease in the amount of free benzoic acid excreted. In a discussion of the question as to the magnitude of hippuric acid formation in the animal body, Wiechowski states that the synthesis bears no relationship either to the weight

[12] A. van de Velde and B. J. Stokvis: *ibid.*, xvii, p. 189, 1883.

of the animal, or to the amount of protein catabolized. He concludes that it depends largely upon the individual power of synthesis, and also upon the size of the dose administered; one large dose, given in fractions during many hours, causes less non-conjugated benzoic acid to appear in the urine, than when the same dose is introduced at once.

Considerable light was thrown by Seo[13] on the cause of the rapid decomposition of hippuric acid in urines. He shows that this is accomplished, not by a ferment, but by bacteria already present in the urine before it leaves the bladder. *Staphylococcus albus* and *Staphylococcus aureus* are, according to Seo, responsible for the decomposition of hippuric acid in urine. Streptococci possess the same power, but not *coli* and other bacteria.

Methods.

With the object of testing the stability of hippuric acid, we allowed different samples of urine to stand twenty-four or more hours at ordinary and at low temperature, with the addition of acid and without. An examination of the figures in Table V shows that the decomposition of hippuric acid in an alkaline urine at room temperature is very large. The amount of free benzoic acid is increased from two to five times in forty-eight hours. The same takes place in a urine slightly acid to litmus, obtained from animals kept on a milk and egg diet.[14] The splitting of hippuric acid is considerable too, when the urine is kept in the refrigerator. It is, however, perfectly safe to collect the urine in a bottle charged with 10 cc. of about 2 per cent nitric acid, as our figures in Table V demonstrate.

Having in mind the possibility of the splitting of hippuric acid while the urine is being collected, we catheterized the animals twice a day, and so regulated their liquid intake, that no urine was voided in the cage. In some of the experiments, however, where the animals did void urine, it was collected in acid, as described. The urine was analyzed immediately after catheterization. Our figures represent, therefore, the amount of free benzoic acid actually eliminated by the animal. Male rabbits only were used in this work. They were fed either on cabbage

[13] Y. Seo: *ibid.*, lviii, p. 75, 1908.

[14] It would appear then, that Wiechowski's assumption, that there is no decomposition of hippuric acid in urines of rabbits fed on oats, is untenable; since such urines are even less acid than the kind secured by feeding milk and egg.

or carrots, or a mixture of milk and egg,[15] given by stomach tube. The latter food was tolerated by all animals; they maintained their weight and appeared to be in good health. Benzoic acid[16] was analyzed according to the method described by us;[17] hippuric acid was determined by the method of Folin and Flanders.[18]

EXPERIMENTAL.

Rabbit A. (Table I.) The animal, weighing 1810 gm., received the first day 1.44 gm. of benzoic acid, 0.79 gm. per kilo, in the form of sodium benzoate subcutaneously.[19] The amount of free benzoic acid excreted in the first five hours was larger than for the same number of hours on any other day in which a similar dose was given; hippuric acid synthesis represented 89 per cent of the total benzoic acid excreted. In the next eighteen hours, only 15 mg. of free benzoic acid appeared in the urine, and the synthesis was as high as 97.4 per cent. The same dose of benzoic acid was administered the second day; non-conjugated benzoic acid excretion in the first six hours was considerably smaller, less than half of what was found on the first day. On the third and fourth days, the same dose was given, but the animal was on a high protein diet of milk and egg, instead of vegetables. The urine is acid to litmus and a diminution of free benzoic acid is noted, due to this special diet. The eighteen hour period of the fourth day is marked by the absence of free benzoic acid. The synthesis of hippuric acid on these days was practically complete, reaching 98.3 per cent. On the fifth day, the dose of benzoic acid was increased to 1.3 gm. per kilo, but the synthesis was as good as when the animal received only 0.88 gm. per kilo; this was due to the high protein diet. The animal was allowed to rest for two days, being fed on vegetables. On the eighth and ninth days the same moderate dose was given as on the first four days. The amount of free benzoic acid was almost the same as on the second day, the synthesis being a little over 96 per cent. On the tenth day, a larger dose of benzoic acid was given, 1.37 gm. per kilo, and the synthesis of hippuric acid was again high—96.6 per cent. We note generally a better ability to synthesize, even on a vegetable diet, as the experiment proceeds. On the eleventh day, the rabbit received a very large dose of benzoic acid, 1.66

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[15] The mixture was prepared by beating up one egg in 200 cc. of milk, and adding 20 gm. of cane sugar. Of this mixture, 100 cc. were given each day in two equal portions.

[16] Amounts of benzoic acid less than 0.020 gm., which we found and recorded, are not claimed to be absolute, having only a relative significance.

[17] G. W. Raiziss and H. Dubin: this *Journal*, xx, p. 125, 1915.

[18] O. Folin and F. F. Flanders: *ibid*, xi, p. 257, 1912.

[19] The method of administering benzoic acid, whether subcutaneously or *per os*, had no bearing on the results obtained.

TABLE I.

Rabbit A.

Day	Period	Free benzoic acid excreted in urine	Total benzoic acid excreted in urine	Benzoic acid as hippuric acid		Weight	Benzoic acid given	Dose per kilo	Diet
				Gm.	Per cent of total				
	hrs.	*gm.*	*gm.*			*gm.*	*gm.*	*gm.*	
1	5	0.089	0.808	0.719	**87.0**				
	18	0.015	0.563	0.552	**97.4**	1810	1.44	0.79	Cabbage.
	Total	0.104	1.375	1.271	**92.6**		subcutaneously		
2	6	0.036	0.820	0.784	**95.6**				
	18	0.015	0.610	0.595	**97.7**	1659	1.44	0.87	Cabbage.
	Total	0.051	1.430	1.379	**96.8**		subcutaneously		
3	6	0.027	0.887	0.860	**96.9**				
	18	0.012	0.534	0.522	**97.8**	1645	1.44	0.88	Cabbage.
	Total	0.039	1.421	1.382	**98.2**		subcutaneously		
4	6	0.024	0.822	0.798	**97.2**				
	18	None	0.577	0.577	**100.0**	1645	1.44	0.88	Milk and egg.
	Total	0.024	1.399	1.375	**98.3**		*per os*		
5	6	0.033	1.205	1.172	**97.3**				
	18	None	0.828	0.828	**100.0**	1605	2.12	1.30	Milk and egg.
	Total	0.033	2.033	2.000	**98.4**		*per os*		
8	5	0.022	0.815	0.793	**97.3**				
	19	0.030	0.561	0.531	**94.7**	1575	1.44	0.91	Milk and egg.
	Total	0.052	1.376	1.324	**96.4**		*per os*		
9	5.5	0.042	0.816	0.774	**94.8**				
	18.5	None	0.562	0.554	**98.8**	1600	1.44	0.90	Carrots.
	Total	0.042	1.378	1.328	**96.4**		*per os*		
10	6	0.045	1.130	1.085	**96.1**				
	18	0.023	0.903	0.880	**97.4**	1550	2.12	1.37	Carrots.
	Total	0.068	2.033	1.965	**96.6**		*per os*		
11	6	0.301	1.310	1.009	**77.0**				
	18	0.074	0.838	0.764	**91.6**	1535	2.54	1.66	Carrots.
	Total	0.375	2.148	1.773	**82.6**		*per os*		
12	6	0.286	1.250	0.964	**77.0**	1477	2.54 *per os*	1.72	Carrots.

gm. per kilo, which is almost the lethal dose.[20] The condition of the animal changed, showing the effects of benzoic acid intoxication. We see here also a decrease in the synthetic power; in the first six hours it fell to 77 per cent, rising in the next eighteen hours to 91.6 per cent; the average for the day was 82.6 per cent. Administration of another large dose on the following day proved fatal, though a six hour period was obtained. In this period, the synthesis was 77 per cent. We note that even when the animal was given a fatal dose the synthesis was high.

TABLE II.

Rabbit B.

DAY	PERIOD	FREE BENZOIC ACID EXCRETED IN URINE	TOTAL BENZOIC ACID EXCRETED IN URINE	BENZOIC ACID AS HIPPURIC ACID		WEIGHT	BENZOIC ACID GIVEN	DOSE PER KILO	DIET
				Gm.	Per cent of total				
	hrs.	*gm.*	*gm.*			*gm.*	*gm.*	*gm.*	
1	6*	—	—	—	—				
	18	0.015	0.710	0.695	98.0	2085	1.69	0.81	Milk and egg.
	Total	—	—	—	—		*per os*		
2	6*	—	—	—	—				
	18	0.012	0.508	0.496	97.8	1875	1.69	0.90	Milk and egg.
	Total	—	—	—	—		*per os*		
3	6	0.008	0.728	0.720	99.0				
	18	None	0.437	0.437	100.0	1782	1.44	0.81	Milk and egg.
	Total	0.008	1.165	1.157	99.5		subcutaneously		
4	6	None	0.768	0.768	100.0				
	18	0.008	0.188	0.180	95.7	1782	1.44	0.81	Milk and egg.
	Total	0.008	0.956	0.942	98.5		subcutaneously		

* No determinations made.

Rabbit B. (Table II.) This animal was kept for a few days before and during the entire experiment on a milk and egg diet. Moderate amounts of benzoic acid, from 0.8 to 0.9 gm. per kilo, were given *per os* and subcutaneously. The rabbit did not tolerate benzoic acid well and suffered from diarrhea. No estimations of free benzoic acid were made for the six hour periods of the first two days, because the urine was contaminated by feces, and under these conditions the results would have been high. In the next periods this was avoided by regulating the liquid intake,

[20] Wiener found the lethal dose to be 1.7 gm. per kilo.

so that the rabbit did not void any urine, it being collected by catheter. It may be seen from Table II that in all cases only traces of free benzoic acid were found in the urine. The synthesis of hippuric acid was practically quantitative. These results fully corroborate those found in the previous experiments; namely, that the administration of moderate amounts of benzoic acid results in a quantitative synthesis.

Rabbit C. (Table III.) The object of this experiment was to study the synthesis of hippuric acid on the administration of very large amounts of benzoic acid. The animal showed considerable tolerance for the acid, signs of intoxication appearing only when nearly fatal doses were given. On the first day 0.88 gm. of benzoic acid per kilo was administered, the rabbit being fed on carrots. The synthesis was, as in our previous experiments, almost complete, reaching 95.0 per cent. With an increased dose of benzoic acid, 1.02 gm. per kilo, on the following day, the synthesis dropped in the first six hours to 87.6 per cent, rising to a higher level, 96.4 per cent in the next eighteen hours. On the third day a still higher dose was given, 1.28 gm. per kilo, causing the synthesis to drop to 87.7 per cent as an average for the day. The dose was increased on the fourth day to 1.53 gm. per kilo, almost a fatal dose. The rabbit, however, remained in comparatively good condition. The synthesis in the first six hours was 82.2 per cent; in the next eighteen hours, 51.8 per cent. The latter figure was the lowest obtained in our experiments and can hardly be brought into accord with other data, since we usually have had lower synthesis during the first six hours, and higher in the subsequent eighteen hour period; in this one case the results are reversed. To be sure that the discrepancy was not due perhaps to some error in the analysis, it was repeated, with the same result.

In the following days our intention was to study the influence of a high protein intake on the synthesis of hippuric acid. On the fifth day the rabbit received no benzoic acid, his food being changed to a milk and egg régime. On subsequent days very large doses of benzoic acid were given and, due to the protein food, a markedly high synthesis resulted. In the six hour periods we see that the synthesis is 96 to 97 per cent, while the eighteen hour periods are characterized in two cases by a complete synthesis, no free benzoic acid being found. Special attention was directed to the eighth day, on which 1.6 gm. of benzoic acid per kilo, nearly a fatal dose, were given, the animal showing signs of severe intoxication. The synthesis on this day, however, was almost quantitative, reaching 95.8 per cent. From here on till the twenty-third day the animal was fed on vegetables, no benzoic acid being given. The same is true of the twenty-fourth, twenty-fifth, and twenty-seventh days. On the twenty-third, twenty-sixth, and twenty-eighth days, the animal received large doses of benzoic acid. As in the previous experiments, this animal also acquired a greater power of synthesis, showing 92.3 per cent as an average for the twenty-eighth day, against 69.3 per cent for the fourth day; in both cases the same amount of benzoic acid was administered and the same diet given.

TABLE III.

Rabbit C.

Day	Period	Free benzoic acid excreted in urine	Total benzoic acid excreted in urine	Benzoic acid as hippuric acid		Weight	Benzoic acid given	Dose per kilo	Diet
				Gm.	Per cent of total				
	hrs.	*gm.*	*gm.*			*gm.*	*gm.*	*gm.*	
1	6	0.045	0.806	0.761	**94.5**				
	18	0.023	0.542	0.519	**95.8**	1660	1.44	0.88	Carrots.
	Total	0.068	1.348	1.280	**95.0**		*per os*		
2	6	0.105	0.833	0.728	**87.6**				
	18	0.028	0.768	0.740	**96.4**	1660	1.69	1.02	Carrots.
	Total	0.133	1.601	1.468	**91.7**		*per os*		
3	6	0.124	1.030	0.906	**88.0**				
	18	0.105	0.810	0.705	**86.8**	1660	2.12	1.28	Carrots.
	Total	0.229	1.840	1.611	**87.7**		*per os*		
4	6	0.226	1.260	1.034	**82.0**				
	18	0.452	0.939	0.487	**51.8**	1660	2.54	1.53	Carrots.
	Total	0.678	2.199	1.521	**69.3**		*per os*		
6	6	0.045	1.204	1.159	**96.2**				
	18	0.023	0.820	0.797	**97.2**	1690	2.12	1.25	Milk and egg.
	Total	0.068	2.024	1.956	**96.8**		*per os*		
7	6	0.060	1.189	1.129	**95.0**				
	18	None	0.828	0.828	**100.0**	1630	2.12	1.30	Milk and egg.
	Total	0.060	2.017	1.957	**97.2**		*per os*		
8	6	0.098	1.244	1.146	**92.3**				
	18	None	0.994	0.994	**100.0**	1590	2.54	1.60	Milk and egg.
	Total	0.098	2.140	2.140	**95.8**		*per os*		
23	6	0.090	0.888	0.798	**90.0**				
	18	0.081	1.270	1.189	**93.6**	1785	2.54	1.42	Cabbage.
	Total	0.171	2.158	1.987	**90.8**		*per os*		
26	6	0.286	0.768	0.482	**62.8**				
	18	0.015	1.350	1.335	**98.8**	1760	2.54	1.44	Cabbage.
	Total	0.301	2.118	1.817	**85.8**		*per os*		
28	6	0.120	0.777	0.657	**84.6**				
	18	0.045	1.340	1.295	**96.6**	1735	2.54	1.46	Cabbage.
	Total	0.165	2.117	1.952	**92.3**		*per os*		

Rabbit D. (Table IV.) This experiment corroborates our previous finding; namely, that the synthesis of the first day is smaller than that of the subsequent days. In the first day, the synthesis was 77.3 per cent, against 84.8 per cent for the fourth day. Milk and egg diet, as previously shown, brings about also in this case a considerably higher synthesis, 93.2 per cent. Altogether this animal exhibited a somewhat smaller power of hippuric acid synthesis than that shown by other rabbits with which we worked.

TABLE IV.

Rabbit D.

Day	Period	Free benzoic acid excreted in urine	Total benzoic acid excreted in urine	Benzoic acid as hippuric acid: Gm.	Benzoic acid as hippuric acid: Per cent of total	Weight	Benzoic acid given	Dose per kilo	Diet
	hrs.	*gm.*	*gm.*			*gm.*	*gm.*	*gm.*	
1	6	0.340	0.758	0.418	**55.2**				
	18	0.142	1.370	1.228	**89.6**	2200	2.54	1.15	Milk and egg.
	Total	0.482	2.128	1.646	**77.3**		*per os*		
3	24	0.355	2.100	1.745	**83.2**	2250	2.54 *per os*	1.13	Milk and egg.
6	6	0.086	0.703	0.617	**87.8**				
	18	0.053	1.325	1.272	**96.0**	2090	2.54	1.22	Milk and egg.
	Total	0.139	2.028	1.889	**93.2**		*per os*		
8	6	0.228	0.693	0.465	**67.2**				
	18	0.076	1.318	1.242	**94.4**	2000	2.54	1.27	Cabbage.
	Total	0.304	2.011	1.707	**84.8**		*per os*		

DISCUSSION.

Experiments performed on four animals indicate clearly that normal rabbits have a strong tendency to eliminate benzoic acid totally as hippuric acid. On an ordinary vegetable diet, when moderate doses are given, not exceeding 1 gram per kilo, the synthesis is almost quantitative, 95 to 98 per cent. Such results, however, can be obtained only when particular care is given to the collection of urine, avoiding any splitting of hippuric acid. In this respect the results of our work differ from those of others. They contradict, for example, the statement made by Wiechowski[1] that the synthesis of hippuric acid is not quantita-

tive even on small doses of benzoic acid. In several cases he found the synthesis to be from 75 to 80 per cent on doses of 0.8 of a gram per kilo. Our results are entirely different from those obtained recently by Kingsbury and Bell.[9] The synthesis in some of their normal rabbits is as low as 28.6 and 32.9 per cent, while the total dose given was only 0.63 gram. Our findings are in accord with Dakin's,[21] in which he shows a practically complete synthesis of hippuric acid, on administering benzoic acid to men in doses of 5 to 10 grams per day. Our figures also support the assumption of some workers, that the elimination of free benzoic acid

TABLE V.

Benzoic acid in urine. 1st analysis	Time elapsing	Benzoic acid in urine. 2d analysis	Benzoic acid from splitting of hippuric acid	Mode of preservation of urine	Reaction of urine to litmus	Diet
gm.	*hrs.*	*gm.*	*gm.*			
0.015	24	0.116	**0.101**	Kept in refrigerator	Acid	Milk and egg.
None	48	0.090	**0.090**			Milk and egg.
0.089	48	0.187	**0.098**		Alkaline	Cabbage.
0.022	48	0.105	**0.083**			Carrots.
0.030	48	0.121	**0.091**			
None	48	0.090	**0.090**			
0.105	24	0.294	**0.189**	Room temperature		Cabbage.
0.286	24	0.437	**0.151**			
0.015	24	0.015	None	10 cc. of 2% nitric acid at room temperature		
0.120	24	0.120	None			
0.045	24	0.045	None			

in rabbits is very small, so that the calculation of glycocoll elimination may be made on the basis of total benzoic acid eliminated. It must be borne in mind, however, that this is justified only when the dose of benzoic acid is moderate.

As may be seen from the tables, the synthesis begins to be incomplete as soon as higher doses are administered, but not as much so as reported in some investigations. On moderate doses the animals showed a uniformly high synthesis, while on larger doses individual differences come to light. In all cases we note

[21] H. D. Dakin: this *Journal*, vii, p. 103, 1909-10.

a distinct development of the ability to synthesize hippuric acid. The first day of feeding was marked, as a rule, by the highest elimination of non-conjugated benzoic acid.

A marked influence on the completeness of synthesis was shown by the high protein diet. On milk and egg, free benzoic acid, even after the administration of very large doses, appears in the urine in only minute quantities, being often not present at all. On this diet the synthesis reaches its highest mark.

There are different factors which must be considered in an attempt to explain the rôle of protein diet in hippuric acid synthesis. It was emphasized in this work how readily hippuric acid is split in an alkaline urine at room or body temperature, or even that of the refrigerator. By precautions already mentioned, we did away with the possibility of this happening, once the urine was voided; but we could not, of course, prevent this from taking place while the urine was still in the bladder. One of the possible causes of the diminution in the amount of free benzoic acid excreted on a protein diet is that such diet causes the secretion of acid urine, and this acidity prevents the splitting of conjugated benzoic acid.

This possibility, however, does not account for all of the free benzoic acid excreted on a vegetable diet; for if this were so, then the amount of free benzoic acid in the eighteen hour periods should be greater than, or at least equal to, that found in the six hour periods. Our figures show the contrary to be true, so that another reason must be sought. A tentative explanation is the following: When a very large amount of benzoic acid is introduced in one dose, as we did, the synthetic power of the animal body is overtaxed, and it takes time until the cells can take care of all the benzoic acid. During this time some of it escapes uncombined. On a milk and egg diet, which is richer in protein than vegetable food, more glycocoll is available at all times, hence the ability of the animal to synthesize in the first few hours is not overburdened. Undoubtedly there are other causes of the appearance of non-conjugated benzoic acid in the urine, but the experimental evidence obtained in this work is not sufficient to permit of further discussion.

SUMMARY.

1. The synthesis of hippuric acid in rabbits is practically quantitative, when no more than 1 gram of benzoic acid per kilo is given.

2. Administration of larger doses, while the animal is on a vegetable diet, causes some of the benzoic acid to appear in the urine uncombined, the synthesis ranging from 80 to 90 per cent.

3. A milk and egg diet exerts a favorable influence on the synthesis, making it as high as 96 per cent on doses which are nearly fatal. In many cases, under such conditions, no free benzoic acid whatsoever was found in the urine.

4. The first day of benzoic acid feeding shows less synthesis than subsequent days. There is an increase of the ability to synthesize after repeated administration of benzoic acid.

5. The largest amount of non-conjugated benzoic acid appears in the urine in the first six hours after feeding.

6. Work in which estimations of non-conjugated benzoic acid are involved must be done only on urines properly treated in order to avoid splitting of hippuric acid. A convenient and reliable method is to collect the urine in a bottle containing dilute nitric acid.

ON THE WALDEN REARRANGEMENT IN THE HEXOSES.

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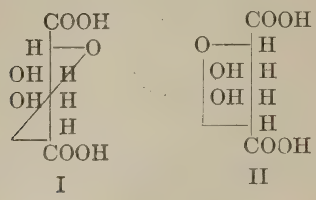
It is known from the work of Fischer and Tiemann that on treatment of glucosamine with nitrous acid two acids are formed, depending on the mode of procedure. Chitonic acid is formed when the amino sugar is deaminized and subsequently oxidized with bromine; chitaric acid when, prior to deamination, the sugar is converted by means of bromine into glucosaminic acid. The two hexonic acids are isomeric and yield on further oxidation isosaccharic and epi-isosaccharic acids respectively. In an earlier paper Fischer[1] explained this isomerism as due to as yet undetermined differences in the configuration; in a more recent paper[2] he referred to the formation of the two substances as an instance of Walden's rearrangement, thus inferring that one of the acids is derived from glucosamine, the other from mannosamine. This view was based on theoretical considerations and lacked experimental support. The missing evidence has recently been furnished by the observations made on the newly discovered amino hexose, chondrosamine.[3] Two isomeric acids, *l*-chondrosic (I) and *l*-epichondrosic (II), were also obtained from this sugar, depending on the order of oxidation and deamination. It was further demonstrated that these two acids correspond to two epimeric sugars: *l*-altrosamine and *l*-allosamine.

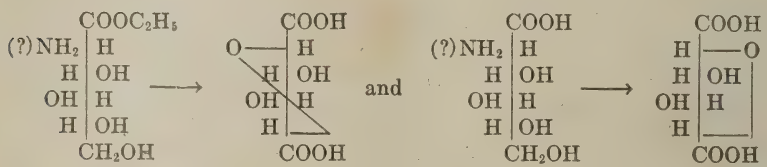
[1] E. Fischer and E. Andreae: *Ber. d. deutsch. chem. Gesellsch.*, xxxvi, p. 2587, 1903.

[2] E. Fischer: *Ann. d. Chem.*, ccclxxxi, p. 123, 1911.

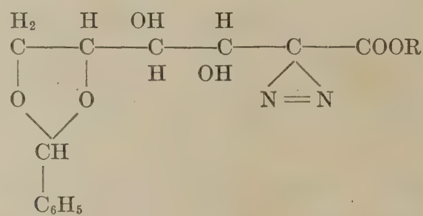
[3] P. A. Levene and F. B. La Forge: this *Journal*, xx, p. 433, 1915.



Furthermore, Fischer and his coworkers[4] have shown that α-amino-acids and their esters, on treatment with nitrous acid, do not yield the same but yield stereoisomeric hydroxy acids. Similarly the α-amino-tetrahydroxycaproic acids and their esters yield two epimeric α-α_1-anhydro-tetrahydroxyadipic acids:



Thus convincing evidence was advanced in favor of the assumption that in either the hydroxy amino-acid or its ester, upon treatment with nitrous acid, the groups attached to the α-carbon atom undergo the Walden rearrangement. The rearrangement is brought about undoubtedly by a complex system of reactions. Fortunately, in the course of this work it was possible to isolate an intermediate substance, the diazo derivative, obtained by the action of nitrous acid upon monobenzalglucosaminic ethyl ester. On the basis of the work of Curtius[5] the substance has the following structure:



[4] Fischer: *Ann. d. Chem.*, cclxxxi, p. 123, 1911; cclxxxvi, p. 374, 1912.
[5] T. Curtius: *Jour. f. prakt. Chem.*, xxxix, p. 107, 1889.

In this phase of the reaction the α-carbon atom loses its asymmetric character. In the transformation of a diazo acid into a hydroxy acid the formation of an equal mixture of the two epimeric forms is to be expected. This does not happen. Only one acid is formed. Fischer,[6] Werner,[7] and Nef[8] have offered theories in order to explain similar phenomena. Nef accepted a rigid difference in the four valencies of the carbon atom and expressed it in the following way:

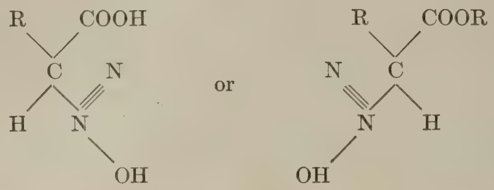
Fischer and Werner assumed that in every reaction of substitution the place of the entering group is determined not by the position of the group being replaced, but by the nature of the remaining three groups and of their respective affinities.

The hypothesis of Nef does not provide an explanation of the phenomenon of the Walden rearrangement, and those of Fischer and of Werner assume that the asymmetric carbon retains its asymmetric nature through all phases of the reaction.

It seemed to us possible that the intermediate reactions which lead to Walden's rearrangement are not identical in every instance. The hypotheses of Fischer and of Werner may be all sufficient for the understanding of the mechanism of the transformation of a halogen acid into a hydroxy acid. The transformation of an amino-acid into a hydroxy acid, or of an amino-acid into a halogen acid, on the other hand, may proceed in a different manner. For the interpretation of the reactions of the second type one may assume that the formation of the diazo derivative is followed by another intermediary phase in which one of the nitrogen atoms becomes detached from the carbon atom, while the other still remains united with it. The nitrogen atom to be detached first is determined by the nature of the other groups in combination with the asymmetric carbon atom. For instance,

[6] Fischer: *Ann. d. Chem.*, ccclxxxi, p. 123, 1911; ccclxxxvi, p. 374, 1912.
[7] A. Werner: *Ber. d. deutsch. chem. Gesellsch.*, xliv, p. 873, 1911.
[8] J. U. Nef: *Jour. Am. Chem. Soc.*, xxx, p. 645, 1908.

one may accept the intermediary formation of the following two structures:



This possibility is supported by the fact that aliphatic diazo acids are readily reduced to amino-acids. Attempts are now in progress to repeat the reduction experiments of Curtius on the diazo hexosaminic acids.

In principle the hypothesis advanced here does not differ from that of Fischer. Both admit the existence of the universally accepted form of static asymmetry of the carbon atom; both also admit the possibility that in certain conditions the formation of the latter form is preceded by a phase in which the asymmetry is expressed only in an uneven distribution of forces between the four valencies, and that the uneven distribution of energy is determined by the character of the groups attached to the carbon atom.

EXPERIMENTAL.

Monobenzalglucosaminic acid ethyl ester hydrochloride.

2.2 grams of finely powdered glucosaminic acid were suspended in 15 cc. of 99.7 per cent ethyl alcohol, 3 cc. of pure benzaldehyde added, and dry hydrochloric acid passed in without cooling for five minutes. After standing a short time the contents of the beaker had become filled with a mass of crystals. An equal volume of dry ether was stirred in and the product allowed to stand for a few hours at a low temperature. The crystals were then filtered off, washed with dry ether, and dried in a desiccator over potassium hydroxide. The yield was 2.65 grams. Experience showed that it was not advantageous to use more than 3 grams of glucosaminic acid for the experiment. For analysis the thoroughly dried substance was recrystallized from absolute alcohol. It crystallizes in long colorless needles which melt at 167–8° (uncorrected) with strong gas evolution.

0.1583 gm. of substance gave 5.65 cc. N, at 22°, 764 mm.
0.1108 gm. of substance gave 0.2090 gm. CO_2 and 0.0637 gm. H_2O.

	Calculated for $C_{15}H_{22}O_6NCl$:	Found:
N	4.04	4.04
C	51.80	51.50
H	6.34	6.39

Diazobenzalglucosaminic acid ethyl ester.

15 grams of benzalester hydrochloride, which had been completely freed from hydrochloric acid by keeping in a desiccator over potassium hydroxide, were dissolved in 400 cc. of water, the solution was cooled to 0°, and 5 grams (about 1.5 mols.) of sodium nitrite were added. After the sodium nitrite had dissolved about 2 cc. of 50 per cent acetic acid were added. Almost immediately separation of a nearly insoluble substance began and within a few minutes the whole volume of the solution was filled with a slightly yellowish, semi-crystalline material. No appreciable gas evolution took place during the process. The product was filtered off with suction and washed with water until free from inorganic salts. When dried it appears as a light yellow powder easily soluble in alcohol, ether, and most organic solvents. It is insoluble in water; it reacts with aqueous mineral acids, and with bromine and iodine with strong gas evolution.

0.1828 gm. of substance gave 14 cc. N, at 22°, 763 mm. (Dumas).

	Calculated for $C_{15}H_{18}O_6N_2$:	Found:
N	8.69	8.66

In dry ethereal solution the product is decolorized by addition of a dry ethereal solution of hydrochloric or hydrobromic acid with vigorous evolution of nitrogen. The resulting solution probably contains as the reaction product the benzal compound of gluconic or mannonic acid ester in which the hydroxyl group on the α-carbon atom is substituted with chlorine or bromine. Upon concentration of the ethereal solution some benzaldehyde is cleaved off; complete elimination of the benzal group is accomplished by short warming on the water bath of the aqueous solution which contains a slight excess of hydrobromic acid. Benzaldehyde and benzoic acid were removed from the aqueous

solution by shaking with ether, and the free bromine was removed by shaking with silver carbonate. The silver was removed from the filtrate with hydrogen sulphide and the solution concentrated in vacuum. The resulting product was a light colored syrup of strong acid properties containing fixed bromine which may be removed by heating with potassium hydroxide or silver oxide in aqueous solution. The substance has not yet been obtained in crystalline form.

XYLOHEXOSAMINIC ACID, ITS DERIVATIVES AND THEIR BEARING ON THE CONFIGURATION OF ISOSACCHARIC AND EPI-ISOSACCHARIC ACIDS.

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The work on chondroitin sulphuric acid[1] is a convincing illustration of the significance of the hexosaminic acids and of the α-α_1-anhydro-tetrahydroxyadipic acids for the progress of our knowledge of hexosamines. Every discovery of a new hexosamine will call for information on the properties and on the physical constants of the acids of these two series. The part to be played by these acids in the development of the chemistry of hexosamines is analogous to that played by the straight hexonic and by the tetrahydroxyadipic acids in that of the hexoses.

These considerations inspired the present work on the preparation of the missing hexosaminic acids and of the corresponding anhydro-tetrahydroxyadipic acids. The present communication deals with the derivatives of xylose. It was hoped through the study of its derivatives to complete our information concerning the details of the configuration of isosaccharic and of epi-isosaccharic acids.

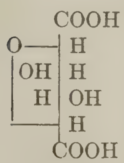
The cyanhydrin synthesis leads from xylose to two epimeric acids: idonic (II) and gulonic (III). In an analogous manner the addition of hydrocyanic acid to xylosimine should lead to two hexosaminic acids: idosaminic (IV) and gulosaminic (V).

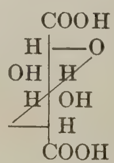
		COOH		COOH		COOH		COOH	
CHO		H	OH	OH	H	H	NH_2	NH_2	H
OH	H	OH	H	OH	H	OH	H	OH	H
H	OH	H	OH	H	OH	H	OH	H	OH
OH	H	OH	H	OH	H	OH	H	OH	H
CH_2OH		CH_2OH		CH_2OH		CH_2OH		CH_2OH	
I		II		III		IV		V	

[1] P. A. Levene and F. B. La Forge: this *Journal*, xx, p. 433, 1915.

In reality the formation of one of the two acids predominates to such an extent that only one can be isolated from the reaction product. It is not possible at the present state of knowledge to be certain whether the predominating substance is (IV) or (V).

When this acid is deaminized with nitrous acid and is then further oxidized by means of nitric acid it gives rise to an α-α_1-anhydro-tetrahydroxyadipic acid. The configuration of this is easily ascertained. Gulosaminic acid, as seen from the graphic formula (V), gives rise to α-α_1-anhydro-saccharic acid (VI), while idosaminic acid (IV) under the same conditions forms α-α_1-anhydro-idosaccharic acid (VII). The first should be identical with the α-α_1-anhydro-saccharic acid corresponding to glucosaminic acid (VIII). In other words, of the four α-α_1-anhydro acids of the saccharic acid series which are obtainable from xylohexosamine and arabohexosamine the two identical in properties correspond to glucosamine and gulosamine.


VI
α-α_1-Anhydro-saccharic.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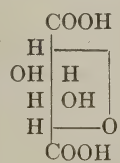
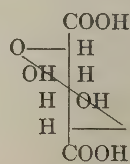
α-α_1-Anhydro-idosaccharic.


VIII
α-α_1-Anhydro-saccharic.


IX
α-α_1-Anhydro-mannosaccharic.

The anhydro acid obtained from xylohexosaminic acid differs in its rotation, its melting point, and in the properties of its acid potassium salt either from isosaccharic or from epi-isosaccharic acids, hence it has the configuration of α-α_1-anhydro-idosaccharic acid (VII).

However, the knowledge of the configuration of an anhydro-tetrahydroxyadipic acid does not carry with it information on the relative position of the amino group in the parent amino-acid, so long as it is known that α-amino-acids in the process of deamination may undergo the Walden rearrangement.

The anhydro-tetrahydroxyadipic acid corresponding to the epimeric xylohexosamine was prepared principally for its bearing on the configuration of isosaccharic and epi-isosaccharic

acids. In a previous communication we pointed out that the present state of information did not permit a decision as to which one of the two was the anhydro-saccharic acid. The conclusion, however, would become obvious after the anhydro-tetrahydroxy-adipic acid corresponding to gulosamine was obtained.

The problem was therefore reduced to the preparation of the epimer of anhydro-idosaccharic acid.

The anhydro-tetrahydroxyadipic acid obtained from glucosamine (for the present it would be more fitting to refer to it as arabohexosamine) is epimeric with the one obtained from glucosaminic acid, and that obtained from chondrosamine is epimeric with the one obtained from chondrosaminic acid. The same method surely might have been applied successfully to xylohexosamine. However, it was thought that the substance could be obtained in a simpler manner. Fischer and his coworkers have shown that amino-acids and their esters do not yield on treatment with nitrous acid the same hydroxy acid, but yield two stereoisomers. It was, therefore, thought that the same method might be applicable to hydroxy amino-acids. In the special instance of xylohexosaminic acid the lactone was employed instead of the ester, since it offered some advantages from the standpoint of preparation. As was to be expected, in place of anhydro-idosaccharic (VII), the epimeric acid (VI) was obtained. The optical rotation, melting point, and the properties of the acid potassium salt were identical with those of epi-isosaccharic acid.

For simplicity of discussion the xylohexosaminic and the corresponding anhydro acids were presented as derived from *d*-xylose (Fischer's nomenclature; according to Rosanoff it should be termed *l*-xylose). The naturally occurring *l*-xylose leads to *l*-gulose, or to *l*-gulosamine, which form on oxidation *l*-saccharic and *l*-anhydro-saccharic acids respectively.

It may also be mentioned that the ethyl ester of xylohexosaminic acid was obtained. The substance was isolated and identified as the dibenzalxylohexosaminic ester hydrochloride. It was originally prepared for the purpose of converting it into anhydro-saccharic acid. Since this was obtained from the lactone of the hexosaminic acid, further work on the ester was abandoned. Work on lyxose derivatives is now in progress.

RÉSUMÉ.

1. From xylose, by treatment with ammonia and subsequently with hydrocyanic acid, a xylohexosaminic acid was obtained.

2. On oxidation following deamination this acid yields α-α_1-anhydro-idosaccharic acid.

3. The lactone of the amino-acid under the same treatment leads to the α-α_1-anhydro-saccharic acid.

4. Isosaccharic acid has the configuration of α-α_1-anhydro-mannosaccharic acid, while epi-isosaccharic acid is the α-α_1-anhydro-saccharic acid, and consequently,

5. Chitose and chitonic acid have the configuration of α-δ-anhydro-mannose and of α-δ-anhydro-mannonic acid respectively; chitaric acid has that of α-δ-anhydro-gluconic acid.

EXPERIMENTAL.

Xylohexosaminic acid.

75 grams of xylosimine[2] were covered with 60 cc. of water, and 20 cc. of 80 per cent hydrocyanic acid were added. The mixture was warmed for about three-quarters of an hour to 35–40° and then allowed to stand at ordinary temperature for about one and a half hours, or until the contents of the flask had taken on a syrupy consistence and had darkened considerably. It was then cooled down in ice water and dissolved in 350 cc. of concentrated hydrochloric acid which had also previously been cooled to 0°, and hydrochloric acid gas passed in at room temperature until the solution was saturated. After this it was allowed to stand for about twenty hours at room temperature and finally warmed for about one and a half hours on the water bath. The solution was concentrated in vacuum to a syrup and mixed with 2 liters of absolute alcohol which caused the separation of a large part of the ammonium chloride together with a black tarry material. These were filtered off on a folded filter, and the alcoholic solution was again concentrated in vacuum to a thick syrup. (The solution was of a dark brown color at first, but became nearly colorless through the subsequent operations.) The syrup was

[2] C. A. Lobry de Bruyn and F. H. Van Leent: *Rec. d. trav. chim. d. Pays-Bas*, xiv, p. 144, 1895.

dissolved in about 500 cc. of water, and after addition of sufficient hot concentrated barium hydrate solution to render it strongly alkaline, distilled in vacuum until all the ammonia was removed. At times it was necessary to dilute the residue with water and to repeat the evaporation. The final solution was then diluted to about 1 liter with water and the barium removed with a slight excess of sulphuric acid. Without filtering from barium sulphate, sulphuric and hydrochloric acids were removed with lead carbonate, hydrogen sulphide was passed through the filtrate, which was finally treated with an excess of silver carbonate, filtered, and the excess of silver removed with hydrogen sulphide. The filtrate was then concentrated in vacuum to about 50–60 cc. Out of this solution the hexosaminic acid crystallizes upon slow addition of about an equal volume of methyl alcohol. The yield of nearly pure substance was 15 grams. For analysis it was purified by solution in about 4 parts of hot water and addition of an equal volume of methyl alcohol. It crystallizes in large colorless prisms which begin to darken above 190° and decompose at about 235° (uncorrected).

0.1335 gm. of substance gave 16.8 cc. amino N, at 18°, 763 mm.
0.1214 gm. of substance gave 0.1648 gm. CO_2 and 0.0760 gm. H_2O.

	Calculated for $C_6H_{13}NO_6$:	Found:
C	36.92	37.01
H	6.66	7.00
N	7.18	7.26

The rotation of the substance was as follows:

$$[\alpha]_D^{30} \text{ after 20 minutes} = \frac{+0.82° \times 2.1542}{1 \times 0.1500} = +11.77°$$

$$[\alpha]_D^{30} \text{ after 40 hours} = \frac{-0.21° \times 2.1542}{1 \times 0.1500} = -3.01°$$

Lactone hydrochloride of xylohexosaminic acid.

7.7 grams of amino-acid were suspended in 75 cc. of 99.5 per cent ethyl alcohol into which hydrochloric acid gas was passed, without cooling, until the alcohol was saturated. At first the substance passed into solution and immediately afterwards a small amount of a white precipitate began to form. This in turn passed into solution as more hydrochloric acid was passed

in. Finally, after the solution had become saturated, crystallization of the lactone began and was complete after the solution had stood in the cold for a few hours. The crystals were then filtered off and washed with absolute alcohol. The yield was practically quantitative, 7.7 grams giving 8 grams of lactone hydrochloride. The substance crystallizes in compact aggregates of prismatic needles grouped together in the form of spheres. When recrystallized from absolute alcohol, in which it is rather sparingly soluble, the crystals appear as long prisms. It melts at 195° (uncorrected), with decomposition and gas evolution, after it has begun to turn brown at about 180°.

0.1910 gm. of substance gave 11.5 cc. N, at 19°, 751 mm.
0.1238 gm. of substance gave 0.0829 gm. AgCl.

	Calculated for $C_6H_{12}NO_5Cl$:	Found:
N	6.60	6.74
Cl	16.25	16.22

Dibenzalxylohexosaminic acid ester hydrochloride.

0.75 gram of amino-acid was suspended in 15 cc. of absolute alcohol and hydrochloric acid gas was passed into the suspension, without cooling, until complete solution had taken place. At this stage 2 cc. of pure benzaldehyde were added and more hydrochloric acid gas was passed into the solution. Very soon the contents of the flask were filled with a mass of crystals. These were filtered off, washed with absolute alcohol, and dried in a desiccator over potassium hydroxide. The yield was 1.2 grams. The completely dried product was recrystallized from a large amount of absolute alcohol. It melted at 217° (uncorrected). The analysis indicates that two benzaldehyde groups have entered into the reaction.

0.2116 gm. of substance gave 6.0 cc. N, at 19°, 760 mm.
0.1297 gm. of substance gave 0.2875 gm. CO_2 and 0.0690 gm. H_2O.

	Calculated for $C_{22}H_{26}NO_6Cl$:	Found:
C	60.65	60.47
H	6.34	6.39
N	3.20	3.25

α-α₁-Anhydro-idosaccharic acid.

9 grams of xylohexosaminic acid were dissolved in 100 cc. of 3 per cent hydrochloric acid and deaminized with 9 grams of silver nitrite. After standing for about twenty-four hours the slight excess of silver was removed with a little hydrochloric acid, the silver chloride filtered off, and the filtrate concentrated in vacuum to about 20 cc. An equal volume of concentrated nitric acid was added and the solution boiled over a small flame for twelve minutes. It was then transferred to a shallow dish and rapidly concentrated on the water bath to a thick syrup. This was taken up in a small amount of water and again evaporated to remove the excess of nitric acid. Crystallization took place spontaneously during the second evaporation. After standing for a short time the crystals were washed from adhering syrup with a mixture of 1 part of dry acetone + 3 parts of dry ether, filtered, and dried in a desiccator. The yield was 3.5 grams. It was recrystallized for analysis by dissolving in 10 parts of dry acetone and adding an equal volume of dry ether. The substance crystallizes under these conditions in long colorless prismatic needles, which melt at 226° (uncorrected), with gas evolution. The product contains 2 molecules of crystal water which may be removed by heating in vacuum at 108°.

I. 0.1220 gm. of substance gave 0.0188 gm. H_2O.
II. 0.1240 gm. of substance gave 0.0192 gm. H_2O.
III. 0.1018 gm. of substance gave 0.0160 gm. H_2O.

	Calculated for $C_6H_8O_7+2H_2O$:	Found: I	II	III
H_2O	15.80	15.53	15.48	15.73

0.1032 gm. of dry substance gave 0.1432 gm. CO_2 and 0.0410 gm. H_2O.

	Calculated for $C_6H_8O_7$:	Found:
C	37.50	37.79
H	4.20	4.45

0.1040 gm. of crystal water containing substance required 9.0 cc. $\frac{N}{10}$ NaOH (calculated 9.1 cc.).

The rotation of the dry substance was as follows:

$$[\alpha]_D^{20} = \frac{-7.6° \times 2.1728}{1.033 \times 1 \times 0.1713} = -93.32°$$

The acid forms an acid potassium salt which is obtained by neutralizing the acid in concentrated aqueous solution with potassium hydrate, adding an equal volume of glacial acetic acid and sufficient alcohol to produce a permanent turbidity. The salt crystallizes in long colorless needles, which, owing to their great solubility in water, are difficult to free from sodium acetate. For this reason no analysis of the product is given.

0.1414 gm. of substance in 2 cc. of water rotated $-6.14°$.

α-α_1-l-Anhydro-saccharic acid (l-epi-isosaccharic acid[3]).

3.5 grams of the above mentioned hydrochloride of xylohexosaminic acid lactone were dissolved in 50 cc. of water and deaminized with 4 grams of silver nitrite. The reaction mixture was kept at 0° for the first five hours and then allowed to stand over night at room temperature. If silver was present in the solution it was removed with a few drops of hydrochloric acid, the silver chloride filtered off, and the filtrate concentrated in vacuum to about 15 cc. An equal volume of concentrated nitric acid was then added and the solution boiled over a small flame for about twelve minutes. It was then evaporated in a flat dish on the water bath to a syrup which was freed from most of the nitric acid by repeating the evaporation after adding a small amount of water. The reaction product was taken up in about 6 cc. of water, neutralized with a strong solution of potassium hydroxide, and allowed to stand fifteen minutes at room temperature, after which an equal volume of glacial acetic acid and about 3 volumes of absolute alcohol were added. Upon standing in the refrigerator from four to five hours crystallization of the acid potassium salt was complete. The yield was 1.6 grams. It was recrystallized from 2 parts of hot water.

0.2314 gm. of substance gave 0.0797 gm. K_2SO_4.

	Calculated for $C_6H_7O_7K + H_2O$:	Found:
K	15.70	15.45

[3] In giving the rotation of the antipode (this *Journal*, xx, p. 444, 1915), we omitted to mention the weight of the solution used, which was 2.1478 grams.

The rotation of the air-dried substance[4] was as follows:

$$[\alpha]_D^{28} = \frac{-2.54° \times 2.1114}{1 \times 0.1400} = -38.09°$$

4 grams of the acid potassium salt were dissolved in about 150 cc. of water and the calculated amount of a 5 per cent solution of lead acetate was added. Crystallization of the lead salt began at once and was complete after about one hour. The lead salt was filtered off with suction, washed with water, and dried at 100°. The yield was 6 grams. The lead salt was suspended in about 100 cc. of water and slightly less than the calculated amount of sulphuric acid added. After standing about one hour on the water bath the lead sulphate was filtered off and the filtrate concentrated in vacuum to a thick syrup which was dissolved in dry acetone and again evaporated to dryness. The syrupy residue, which contained lead salts, was extracted with dry acetone, and the colorless filtrate evaporated in a dish on the water bath to a thick syrup. By repeating the evaporation with acetone a few times the syrup crystallized on cooling without crystal water. It was freed from a small amount of adhering syrup by washing with a mixture of 1 part of amyl alcohol + 2 parts of dry ether. The yield was 1.5 grams. It may be recrystallized by dissolving in a small amount of dry acetone and allowing the solution to evaporate in the air. The substance melts at 163°.

0.1092 gm. of substance gave 0.1514 gm. CO_2 and 0.0410 gm. H_2O.

	Calculated for $C_6H_8O_7$:	Found:
C	37.50	37.80
H	4.20	4.20

The rotation of the substance was as follows:

$$[\alpha]_D^{21} = \frac{-3.08° \times 2.1547}{1.034 \times 0.1657} = -38.79°$$

[4] For comparison we determined the rotation of the same salt of *d*-anhydro-saccharic acid obtained from glucosaminic acid (this *Journal*, xx, p. 442, 1915).

$$[\alpha]_D^{28} = \frac{+2.56° \times 2.1221}{1 \times 0.1410} = +38.53°$$

A METHOD FOR THE DETERMINATION OF CHLORIDES IN SMALL AMOUNTS OF BODY FLUIDS.[1]

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Previous methods for the estimation of chlorides in blood and plasma have required at least 10 cc. of material in order that the error might fall within 1 per cent of the total amount present. Bang's micro-method[2] requires only 100 mg. of blood, but it is hardly accurate to within 5 per cent.

The necessity for frequent chloride determinations on the plasma of individual patients, made simultaneously with other determinations on the same blood samples, led us to seek an accurate method of titrating small amounts of chlorides. As a result, we have recently published[3] an iodometric method for determination of halides in general which permits the titration of as little as 5 cc. of $\frac{N}{10}$ halide solution with an accuracy of 1 part per 1000.

The present paper gives the conditions for the application of this method to body fluids. As the concentration of chloride in blood plasma is about $\frac{N}{10}$, it is possible to titrate the amount in 0.5 cc. with an accuracy of 1 part per 100. In order to leave a margin of safety, we usually take more material, but the agreement between duplicates is then, as a rule, correspondingly closer than the 1 per cent accuracy which is required. The titration is also applicable to urine, ascitic fluid, pleural exudates, gastric juice, etc.

[1] A preliminary report of this method was published in the *Proc. Soc. Exper. Biol. and Med.*, xii, p. 93, 1915.

[2] I. Bang: *Biochem. Ztschr.*, xlix, p. 19, 1913.

[3] F. C. McLean and D. D. Van Slyke: *Jour. Am. Chem. Soc.*, xxxvii, p. 1128, 1915.

Description of the method.

The determination requires two steps: (1) removal of proteins and (2) titration of chlorides.

Coagulation of proteins. Removal of the proteins may be accomplished in two ways, by coagulation or by ignition. Results are identical by both methods, but coagulation is the simpler and is the only means of deproteinizing which we have used in routine work. For coagulation 2 cc. of oxalated plasma (1 cc. may be used if material is limited) are drawn into a 2 cc. pipette which has been calibrated to *contain* 2 ± 0.005 cc. From the pipette the plasma is run into a 20 cc. stoppered volumetric flask which contains 10 cc. of a 10 per cent magnesium sulphate solution. The pipette is rinsed twice by drawing up into it the solution from the flask. Two drops of 50 per cent acetic acid are added, the flask is filled to the mark with water, the contents are mixed by inverting the flask, and heated in a bath to 100° for ten minutes. By keeping the stopper loosely in place evaporation is prevented, and when cool the contents return to their original volume. Ten minutes on the steam bath are sufficient to coagulate the albumin and to distribute the chlorides evenly between the fluid and precipitated albumin.[4] A shorter period of heating may give low values in the filtrate, apparently due to a mechanical carrying down of chloride by protein, but at the end of ten minutes the filtrate contains its proper amount of chloride. The flask is then allowed to cool, and the contents are poured onto about 0.3 gram of blood charcoal in a small beaker, and mixed. (Merck's "Blood Charcoal, Reagent," purified by acid and free from chloride is used. No other form of charcoal has been found to be of service.) After a few minutes the liquid is filtered through a dry folded filter, and a water-clear filtrate obtained. Occasionally a small amount of charcoal passes through with the first few drops of filtrate, but this can be poured back through the same filter, and the filtrate then obtained is absolutely clear and colorless.

[4] The method of coagulation is similar to that of Larsson (*Biochem. Ztschr.*, xlix, p. 479, 1913), but much more rapid. Larsson coagulates at half volume, then dilutes after coagulation, and consequently has to wait two hours for the chloride to diffuse evenly through the coagulum.

Titration of the protein-free filtrate. In brief, the chlorides are precipitated in the presence of nitric acid by standard silver nitrate solution, the silver chloride is removed by filtration, and the excess silver titrated with standard potassium iodide. The titration is performed in the presence of nitrous acid and starch, so that the first drop of iodide in excess of the silver present is changed to free iodine and gives the blue starch-iodine color. The optimum acidity for the end-point is fixed by the addition of trisodium citrate in amount equivalent ($\frac{1}{3}$ mol.) to the free nitric acid present. Under these conditions one drop of excess $\frac{N}{50}$ iodide gives a color perceptible in 150 cc. of solution.

The following solutions are required:

I. An acid M/29.25 solution of silver nitrate, 1 cc. of which is equivalent to 2 mg. of NaCl.[5]

$AgNO_3$	5.812	gm.
HNO_3 (sp.gr. 1.42)	250	cc.
Water to	1000	cc.

In place of silver nitrate, metallic silver, which can be obtained in a high state of purity, may be made the basis of the solution. In this case 3.688 grams of silver are dissolved in the nitric acid and diluted to 1000 cc. We also find it convenient to prepare a stock solution of silver nitrate ten-fold as concentrated as the above, and prepare the M/29.25 solutions from this by dilution. As a check on its accuracy the stock solution may be standardized against a known hydrochloric acid solution by the Volhard method, or gravimetrically.

II. A solution of M/58.5 potassium iodide, 1 cc. of which is equivalent to 1 mg. of NaCl.

KI	3.0	gm.
Water to	1000	cc.

This solution is standardized against the silver solution by adding 5 cc. of the latter to 5 cc. of Solution III and titrating with the iodide solution to the blue end-point. The iodide solution is then diluted to such a degree that 10 cc. are exactly equivalent to 5 cc. of the silver solution.

[5] The total chlorides are, of course, determined by the method. For the sake of convenience, all estimations here reported are calculated as sodium chloride.

III. A solution, for use in the final titration, containing sodium citrate, sodium nitrite, and starch, which substances respectively regulate the acidity, provide an oxidizing agent for the iodide, and serve as indicator.

Sodium citrate ($Na_3C_6H_5O_7 + 5\frac{1}{2}H_2O$)	446	gm.
Sodium nitrite	20	gm.
Soluble starch	2.5	gm.
Water to	1000	cc.

The starch is first dissolved with the aid of heat in about 500 cc. of water. The citrate and nitrite are then added, and the mixture is heated until all is dissolved. The solution, while still hot, is filtered through cotton, the filter washed with hot water, the filtrate allowed to cool, and made up to 1000 cc. Filtration removes insoluble substances occurring chiefly in the nitrite, and cotton filters more rapidly than filter paper. The solution keeps indefinitely. It becomes cloudy on standing, but its efficacy is not impaired.

In detail, the titration of the protein-free plasma filtrate is carried out as follows: Either 10 cc. of the filtrate, containing the chlorides of 1 cc. of plasma, are taken in a pipette for titration, or the filtrate is collected directly in a certified 25 cc. graduated cylinder, where it is measured, so that the entire amount may be taken for titration. In this way 13 to 14 cc. of filtrate, corresponding to 1.3 to 1.4 cc. of plasma, may be obtained for titration. In this case the cylinders must be accurate to within 0.05 cc. Ordinary 25 cc. cylinders are not to be used, as errors in graduation up to 0.2 cc. or more are quite common, and unless certified cylinders are available the use of a 10 cc. pipette is advisable.

After either measuring 10 cc. of the filtrate into a 25 cc. volumetric flask or recording the amount of filtrate obtained in the cylinder, 5 cc. of the acidified M/29.25 silver nitrate solution (Solution I) are added, and the whole is made to the 25 cc. mark with water. This will precipitate up to 10 mg. of NaCl. In samples with high percentage of chloride, only enough filtrate is taken to keep within this limit of 10 mg. Two drops of octyl (caprylic) alcohol are added, and the vessel is stoppered and shaken gently by inverting it several times. Immediate coagulation of the

silver chloride occurs. After allowing five minutes for complete precipitation to occur, the solution is filtered through a dry folded filter, and a perfectly clear and colorless filtrate again obtained. In extracts from whole blood the silver chloride does not coagulate so readily as in plasma extracts, but it yields an equally clear filtrate. In any case some silver chloride may pass through the filter with the first few drops, but it is necessary only to pass them through the same filter again to obtain a filtrate perfectly clear to the eye. Observed in the nephelometer, the turbidity of these filtrates corresponds to only 0.01 mg. or less of sodium chloride.

An aliquot part of the filtrate—20 cc.—is now taken with a pipette for titration. Or, if so desired, the precipitate and filter may be washed several times with water and the entire filtrate titrated. The use of an aliquot part, however, saves time and appears to be equally accurate. Just before titration with the potassium iodide solution (Solution II) one adds a volume of the citrate solution (Solution III) equal to the volume of Solution I represented in the filtrate to be titrated. If, as is usually the case, one has used 5 cc. of Solution I diluted to 25 cc., and taken 20 cc. of the filtrate for titration, one has the equivalent of 4 cc. of Solution I present, and must accordingly add 4 cc. of Solution III. On adding Solution III a slight turbidity appears, which in no way interferes with the end-point.

The potassium iodide solution is then run in from a burette until the blue end-point appears. The first definite blue color is taken as the end-point, and with a slight practice is unmistakable. An additional drop, which may be used as a control, causes such a deep blue color that it is impossible to make an error of more than one drop in titration. Should the end-point be accidentally passed, one may add 1 cc. of Solution I, 1 cc. of Solution III and retitrate, allowing in the calculation for the extra silver nitrate.

The result may be calculated from the following formula, which applies only when 20 cc. of the filtrate from silver chloride are titrated:

$$\text{Grams NaCl per liter} = \frac{12.5\ (8 - \text{cc. KI solution used})}{\text{cc. blood filtrate taken.}}$$

Thus, if 13 cc. of filtrate are obtained and titrated after precipitation of plasma protein, and 1.60 cc. of KI are used in the final titration,

$$\text{Grams NaCl per liter} = \frac{12.5\ (8 - 1.60)}{13} = 6.15.$$

In case only 1 cc. of plasma instead of 2 cc. has been used, the factor 25 replaces 12.5 in the numerator. In case the entire filtrate from the silver chloride instead of 0.8 of it (20 cc.) is taken for the final titration, the formula is $\frac{10\ (10 - \text{cc. KI solution})}{\text{cc. blood filtrate}}$ when 2 cc. of plasma are used, or $\frac{20\ (10 - \text{cc. KI solution})}{\text{cc. blood filtrate}}$ when only 1 cc. is taken.

Accuracy of the method.

The fact that the titration method for chlorides is capable of an accuracy of one part per one thousand has been previously established.[6] The exactness of the above application of the method depends directly on the care with which measurements are made and on the purity of the reagents used. All reagents must, of course, be free from substances precipitated by silver, and are easily tested. As the volumes measured are not large, however, small absolute errors in measurement may cause considerable percentage error in the final result. All glassware must therefore be carefully calibrated by either the weight of water contained (flasks, cylinders, 2 cc. pipettes) or the weight delivered (other pipettes, burettes), and burettes should be used which are capable of being read to 0.02 cc. and which deliver small drops. If these precautions are followed, one should uniformly obtain results as shown in Table I, which are well within a limit of error of 1 per cent.

Control determinations by fusion method.

As stated above, the accuracy of the titration method itself has been established to a degree well beyond the present requirements of blood analysis. In the present application of the

[6] McLean and Van Slyke: *loc. cit.*

method, however, additional manipulation (removal of colloids by coagulation and charcoal) has been introduced in order to eliminate interfering substances, particularly proteins, before precipitating the chlorides. In order to show both that this preliminary manipulation introduces no error, and that it is efficient in removing interfering substances, we have compared

TABLE I.

Comparison of results obtained by coagulation and fusion methods. In each case 5 cc. of M/29.25 $AgNO_3$ solution were used, the volume was made to 25 cc., and 20 cc. of filtrate were titrated with M/58.5 KI solution.

Coagulation					Fusion, 1 cc. sample	
Sample	Dilution	Filtrate titrated	M/58.5 KI used	NaCl per cc. of sample	M/58.5 KI used	NaCl per cc. of sample
		cc.	*cc.*	*mg.*	*cc.*	*mg.*
Normal plasma* I	20:200	12.5	1.95	*6.04*	3.15	*6.06*
" " "		12.5	1.97	*6.03*	3.18	*6.02*
" " "		12.5	1.96	*6.03*		
" " "		12.5	1.98	*6.01*		
" " "		12.5	2.00	*6.00*		
" " II	2:20	13.20	1.72	*5.95*	3.23	*5.96*
" " "	2:20	13.60	1.50	*5.97*		
" " III	2:20	13.10	1.73	*5.98*	3.22	*5.98*
" " "	2:20	12.20	2.12	*6.02*		
" " IV	2:20	14.60	0.82	*6.14*	3.07	*6.16*
" " "	2:20	13.60	1.35	*6.11*		
Nephritic plasma I	2:20	13.80	0.80	*6.52*	2.75	*6.56*
" " II	2:20	13.60	1.24	*6.21*	3.05	*6.19*
Pneumonia plasma	2:20	11.50	2.40	*5.29*	4.20	*5.25*
Diabetic plasma	2:20	14.00	2.35	*5.04*	4.0	*5.00*
Whole blood	2:20	9.90	4.12	*4.90*	4.05	*4.94*
Ascitic† fluid	2:20	14.80	0.42	*6.39*	2.92	*6.35*

* Determination by Volhard method on 15 cc. sample gave *6.06* mg. NaCl per cc. of plasma.
† Determination by Volhard method on 15 cc. sample gave *6.35* mg. NaCl per cc. of fluid.

the results obtained after coagulation with those after fusion, by which all the organic material of the plasma was destroyed. The fusions were done with sodium carbonate and lime, according to the method described by H. Schiff,[7] which accomplishes destruction of the organic matter without the formation of

[7] H. Schiff: *A. Classens Ausgewählte Methoden der analytischen Chemie*, Braunschweig, 1903, ii, p. 763.

TABLE II.

Results of duplicate analyses of plasma by coagulation method. Dilution in each case 1:10, 5 cc. of $AgNO_3$ solution were used, the volume was made to 25 cc., and 20 cc. of filtrate were titrated with KI.

SAMPLE	FILTRATE TITRATED	M/58.5 KI USED	NaCl PER CC. OF PLASMA	DIFFERENCE
	cc.	*cc.*	*mg.*	
1.	13.90	1.40	*5.94*	
	12.35	2.10	*5.97*	0.03
2.	13.05	1.72	*6.01*	
	13.15	1.65	*6.04*	0.03
3.	14.15	1.63	*5.63*	
	13.20	2.05	*5.64*	0.01
4.	13.30	1.54	*6.08*	
	13.85	1.30	*6.05*	0.03
5.	13.10	1.60	*6.11*	
	13.45	1.45	*6.09*	0.02
6.	13.80	1.50	*5.89*	
	13.35	1.72	*5.88*	0.01
7.	14.15	1.22	*5.99*	
	13.0	1.77	*5.99*	0.00
8.	13.20	1.72	*5.95*	
	13.60	1.50	*5.97*	0.01
9.	12.60	1.92	*6.19*	
	13.1	1.50	*6.19*	0.00
10.	13.20	1.72	*5.95*	
	13.60	1.50	*5.91*	0.04
11.	12.5	1.95	*6.04*	
	12.5	1.97	*6.03*	
	12.5	1.96	*6.03*	
	12.5	1.98	*6.01*	
	12.5	2.00	*6.00*	0.04
12.	13.1	1.73	*5.98*	
	12.2	2.12	*6.02*	0.04
13.	14.6	0.82	*6.14*	
	13.6	1.35	*6.11*	0.03
14.	12.75	1.75	*6.13*	
	13.6	1.35	*6.11*	0.02
15.	13.15	1.70	*5.99*	
	12.1	2.18	*6.01*	0.02
16.	12.4	1.81	*6.23*	
	12.10	1.96	*6.24*	0.01
17.	13.90	1.16	*6.15*	
	13.40	1.45	*6.11*	0.04
18.	12.85	1.84	*5.99*	
	12.55	2.0	*5.98*	0.01
19.	12.55	2.0	*5.98*	
	14.45	1.04	*6.02*	0.04

cyanides. One cc. of plasma was mixed in a platinum crucible with 1 gram of a mixture composed of 1 part of Na_2CO_3 to 4 of CaO, dried on an electric hot plate, covered with 0.5 gram of the same fusion mixture, burned for five minutes over a Chaddock burner, and the mixture dissolved in an excess of nitric acid. The solution was made neutral with 60 per cent NaOH, concentrated on the steam bath to about 10 cc., transferred to a 25 cc. volumetric flask, and cooled. 5 cc. of the M/29.25 silver nitrate solution were added, the whole was made to volume, shaken with octyl alcohol, filtered, and 20 cc. of filtrate were titrated as above. Results by this method correspond, as is shown in Table I, very closely to those obtained when proteins are removed by coagulation.

Table II shows results of duplicate analyses by the coagulation method on the same sample. Duplicates agree almost invariably within 0.05 mg. of each other, and usually closer. It is probable that the average error in determining the chlorides in 2 cc. of plasma is much less than 1 per cent.

Application to urine.

The procedure is not superior to the Volhard for ordinary urine titrations. Results fully as accurate as those by the Volhard, however, can be obtained with much less material, and this may make the new method valuable for certain work, such as in comparing the chloride contents of urines collected separately from both ureters, or titrating urines of small animals.

When the urine contains no albumin, it is necessary only to measure 1 cc. into a 50 cc. volumetric flask half filled with water, add 10 cc. of the silver nitrate solution, fill to the mark with water, add 2 to 3 drops of octyl alcohol, shake, and filter. Twenty-five cc. of the filtrate are taken with a pipette, 5 cc. of citrate solution added, and the excess silver is titrated with iodide. 2 (10 − cc. KI) then gives mg. NaCl in 1 cc. of urine, or grams NaCl per liter, and the method as thus applied has a range of 0 to 20 grams per liter. In the case of urines containing albumin, the procedure is the same as for blood, 2 cc. being diluted to 20 cc. with magnesium sulphate, acetic acid, and water, and coagulated. Ten cc. of the filtrate, representing 1 cc. of urine, are then treated as above described.

Tables III and IV give a comparison of results with urines analyzed by the present method and the Volhard titration, respectively, the titrations with the latter being performed in the presence of the silver chloride precipitate.

TABLE III.

Albumin-free urines, not treated with heat and acetic acid, dilution 1:50, including 10 cc. of M/29.25 $AgNO_3$ solution in each case. 25 cc. of filtrate were titrated with KI.

SAMPLE	M/58.5 KI USED	NaCl PER CC. OF URINE	VOLHARD NaCl PER CC. OF URINE
	cc.	*mg.*	*mg.*
Normal urine	3.7	12.6	12.6
Normal urine	3.45	13.1	13.0
Normal urine	4.48	11.04	11.1
Normal urine	5.05	9.9	9.8
Normal urine	7.7	4.6	4.6
Normal urine	5.8	8.4	8.3
Normal urine	5.3	9.4	9.4
	3.35	13.0	13.0
	4.28	11.44	11.5

TABLE IV.

Albuminous urines treated with magnesium sulphate, acetic acid, and heat. 5 cc. of $AgNO_3$ solution were used in each case, the solution was made to 25 cc., filtered, and 20 cc. of filtrate were taken for titration.

SAMPLE	DILUTION	FILTRATE TITRATED	M/58.5 KI USED	NaCl PER CC. OF URINE	VOLHARD NaCl PER CC. OF URINE
		cc.	*cc.*	*mg.*	*mg.*
Nephritic urine + albumin 6.0%	1:20	10.00	4.74	8.16	8.15
Nephritic urine + albumin 5.7%	2:20	12.50	5.2	2.80	2.70
Diabetic urine + albumin trace	1:20	10.00	5.85	5.38	5.30

Other applications.

Whole blood, serum, and ascitic, or pleural fluid are treated in the same way as plasma. For the determination of chlorides in gastric juice, etc., the same manipulation may be carried out, the volume of filtrate used for titration depending on the chloride content.

THE INFLUENCE OF SALICYLATES UPON THE URIC ACID CONCENTRATION OF THE BLOOD.

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(Received for publication, April 24, 1915.)

In a previous communication from this laboratory, in agreement with studies of other workers, it was shown that the well known increased output of uric acid following the administration of atophan was accompanied by a commensurately lowered concentration of uric acid in the blood.[1] From this the conclusion was drawn that atophan induced some change in the permeability of the kidney for uric acid, establishing a lower level of renal retention for this substance, as a result of which there ensued an overflow of uric acid from the blood and tissues into the urine.

It is a common observation that salicylates also provoke a significant increase in the output of uric acid,[2] and it becomes of interest to learn whether we may here likewise be dealing with an altered renal permeability, resulting in a reduction of the uric acid concentration of the blood. In the following tables the data do indeed point to the close resemblance between the action of atophan and salicylates upon the uric acid concentration of the blood. In several instances the effect of the latter drug has, if anything, been even more pronounced, twice causing an almost complete disappearance of uric acid from the blood (Case 1), an observation which we have been unable to make in the case of atophan.

[1] M. S. Fine and A. F. Chace: *Jour. Pharm. and Exper. Therap.*, vi, p. 219, 1914.

[2] See E. W. Rockwood: *Am. Jour. Physiol.*, xxv, p. 34, 1909–10; and G. Pietrulla: *Arch. f. Verdauungskr.*, xix, p. 673, 1913.

The subjects[3] of these studies subsisted upon a purine-free diet whose qualitative and quantitative character remained practically unchanged throughout the periods of observation. The data in the tables are arranged in consecutive periods. The reduction of the uric acid concentration of the blood under the influence of salicylates is manifest, and in most cases the return to the initial concentration took place within a few days after the cessation of the use of the drug. To this, Case 3 forms an exception. Seven days after the end of the treatment there appeared to be no disposition for uric acid to reaccumulate in the blood, and at the end of a further period of thirty-five days the blood uric acid was still considerably below the initial concentration. Apparently, in this case, the kidneys had experienced a more lasting change. It should be noted that an injection of radium bromide had been given early during the aspirin period, but we are not inclined to ascribe the more persistently lowered uric acid concentration to this circumstance.[4]

The method we have employed for determining the uric acid concentration of the blood was, as in our previous studies, that of Folin and Denis, with certain modifications.[5] Final clarification of the blood filtrate was accomplished by means of alumina cream, which was found to be far superior for this purpose to the talc employed in our earlier work. The precipitation of the purines and the subsequent liberation of uric acid were carried out by the admirable method of Benedict.[6] The use of KCN, as described by Benedict, instead of H_2S, has added greatly to the simplicity and accuracy of the determination. In the search for a uric acid standard, we have likewise been greatly aided by Benedict's studies. At first, at his suggestion, we employed a solution of uric acid in a dilute pyridine solution, which is stable for about ten days, and later the excellent permanent standard of uric acid dissolved in a phosphate mixture. We are greatly

[3] We are indebted to Dr. W. G. Lough for assistance in following the clinical course of the cases, and to Mr. Adolph Bernhard for the determinations of uric acid of the urine.

[4] See Fine and Chace: *loc. cit.*, p. 234.

[5] See Fine and Chace: *loc. cit.*, p. 221.

[6] S. R. Benedict and E. H. Hitchcock: this *Journal*, xx, p. 619, 1915. Benedict: *ibid.*, xx, p. 629, 1915.

indebted to Professor Benedict for placing before us the details of his important modifications of the Folin-Denis method and his uric acid standards previous to their publication.

Case 1 (B), rheumatism.

PERIOD	DAYS	URIC ACID OF BLOOD AT END OF PERIOD
		mg. per 100 cc.
Control (no salicylate)		2.0
3 gm. sodium salicylate daily	5	0.0
3 gm. sodium salicylate daily	9	1.4
6 gm. sodium salicylate daily	6	0.4
Control (no salicylate)	5	2.5
6 gm. sodium salicylate daily	4	0.0

Case 2 (W), asthma.

PERIOD	DAYS	URIC ACID OF BLOOD AT END OF PERIOD
		mg. per 100 cc.
Control (no salicylate)		2.7
3 gm. sodium salicylate daily	5	1.6
3 gm. sodium salicylate daily	9	2.0
6 gm. sodium salicylate daily	6	0.8
Control (no salicylate)	7	1.9
6 gm. sodium salicylate daily	2	1.7
Control (no salicylate)	7	2.2

Case 3 (L), arthritis.

PERIOD	DAYS	URIC ACID OF BLOOD AT END OF PERIOD
		mg. per 100 cc.
Control (no salicylate)		3.5*
2 gm. aspirin daily	18	0.7
Control (no salicylate)	7	0.6
Control (no salicylate)	10	1.6
Control (no salicylate)	10	2.0
Control (no salicylate)	15	1.8

*After the blood was drawn, 50 micrograms of radium bromide were injected intravenously.

Case 4 (B), gout.

Period	Days	Uric acid output, daily averages	Uric acid of blood at end of period
		gm.	*mg. per 100 cc.*
Control (no salicylate)	7	0.42	4.4
3 gm. sodium salicylate daily	12	0.63	3.2
8 gm. sodium salicylate daily	2	0.74	1.4
Control (no salicylate)	4	0.39	3.6
Control (no salicylate)	8	0.41	—
6 gm. sodium salicylate daily	10	0.68	1.9
Control (no salicylate)	4	0.31	3.7

Cases 5, 6, 7, and 8.

Case	Period	Days	Uric acid of blood at end of period
			mg. per 100 cc.
5 (F. S.)	Control (no salicylate)		4.0
Hysteria	6 gm. sodium salicylate daily	4	0.8
6 (A. M.)	Control (no salicylate)		2.1
Arthritis	6 gm. sodium salicylate daily	4	0.8
7 (M. E. C.)	Control (no salicylate)		2.0
Arthritis	4 gm. sodium salicylate daily	4	1.4
8 (W. L.)	Control (no salicylate)		3.0
Arthritis	6 gm. sodium salicylate daily	6	2.8
	Control (no salicylate)	9	
	5.3 gm. sodium salicylate daily	9	1.3

After we had offered our preliminary communication upon this subject,[7] our attention was called to a paper by Frank and Pietrulla,[8] which had hitherto escaped our notice. These workers employed the more cumbersome method of determining the uric acid of the blood by isolating the uric acid in crystalline form and titrating with $\frac{N}{100}$ permanganate solution. In several instances the concentration was noted by reference merely to the abundance or paucity of uric acid crystals so obtained. They were nevertheless able to demonstrate a diminution of the blood uric acid after

[7] Fine and Chace: *Proc. Soc. Exper. Biol. and Med.*, xii, p. 95, 1915.

[8] E. Frank and G. Pietrulla: *Arch. f. exper. Path. u. Pharmakol.*, lxxvii, p. 361, 1914.

the use of atophan. Their study of the influence of salicylates comprised three cases, to whom six grams of salicylic acid were given in twenty-four hours. In two cases, no blood uric acid could be demonstrated either before or after the administration of the drug; hence, from these, no conclusions whatever can be drawn. In a third, however, one in which they state uric acid was without doubt originally present, the blood was found to be free from this substance on the morning following the administration of the drug. The results of Frank and Pietrulla here reviewed do not appear to us to be sufficiently clear cut to warrant the drawing of any conclusions.

It has appeared to us to be an interesting circumstance, as we note Frank and Pietrulla have also pointed out,[9] that atophan and salicylates behave almost identically as analgesics and uric acid eliminants; and despite the apparent dissimilarity in these two actions, some relationship is suggested.

We have made a few observations upon the influence of certain other drugs upon the uric acid concentration of the blood. In two cases treated with 5.3 grams of theobromine sodium salicylate daily (one for eleven days and the other for two days) there was, if anything, a slight increase in the concentration of blood uric acid. The same may be said with regard to quinine hydrochloride (2 grams per day for two days in one case), and for colchicum (maximum doses in two cases).

[9] Frank and Pietrulla: *loc. cit.*, p. 371.

THE METABOLISM OF CREATINE AND CREATININE.

SEVENTH PAPER.[1]

THE FATE OF CREATINE WHEN ADMINISTERED TO MAN.

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(Received for publication, April 24, 1915.)

The fate of creatine and creatinine when introduced into the body of man or animals was considered in previous experiments[2] in which these substances had been administered parenterally to rabbits. A small increase was noted in the creatinine excretion after the administration of creatine, indicating a slight conversion of creatine to creatinine. This was believed to possess some little significance as a factor in the metabolic relationship of these two substances. The criticism may be made in general of most previous experiments that too large amounts of creatinine were looked for. Since our experiments leading to this conclusion were largely conducted on rabbits, it seemed desirable to carry out feeding experiments with creatine on man under conditions suitable for detecting this slight increase in the excretion of creatinine.

The writers served as the subjects of these experiments and lived upon meat- and meat extract-free diets for twelve and thir-

[1] The earlier papers of this series are: I. The creatine content of muscle under normal conditions. Its relation to urinary creatinine, this *Journal*, xiv, p. 9, 1913. II. The influence of starvation upon the creatine content of muscle, *ibid.*, xv, p. 283, 1913. III. The influence of carbohydrate feeding upon the creatine content of muscle, *ibid.*, xv, p. 305, 1913. IV. The influence of the administration of creatine and creatinine on the creatine content of muscle, *ibid.*, xvi, p. 169, 1913–14. V. A note on the determination of creatinine and creatine in muscle, *ibid.*, xvii, p. 65, 1914. VI. The non-protein nitrogenous compounds of the blood in nephritis, with special reference to creatinine and uric acid, *ibid.*, xx, p. 391, 1915.

[2] V. C. Myers and M. S. Fine: *ibid.*, xvi, p. 169, 1913–14. References to the literature are given in this paper.

teen days, the first six of which served as a control period. On the seventh day 1 gram of creatine in solution was taken by mouth. The dose was increased 1 gram per day until on the eleventh day 5 grams were taken. We believe that the data on these five consecutive days form a much more reliable comparison with the control period than the data obtained from the ingestion of

TABLE I.

Experiments on V. C. M., November 3 to 18, 1913.

DAY	VOLUME OF URINE	SPECIFIC GRAVITY	CREATININE	DATA ON CREATINE			REMARKS ON DIET, ETC.
				Excreted in urine	Excreted in urine	Taken by mouth	
	cc.		*gm.*	*gm.*	*per cent*	*gm.*	
1	870	1.020	1.46	0			No meat, but meat on preceding day.
2	1000	1.018	1.35	0			
3	470	1.030	1.35	0			
4	590	1.029	1.35	0			
5	575	1.028	1.35	0			
6	570	1.028	1.35	0			
7	910	1.015	1.35	0	0	1.0	
8	1330	1.013	1.38	0.33	16	2.0	Very cold day.
9	850	1.015	1.40	0.72	24	3.0	
10	820	1.016	1.41	1.09	27	4.0	
11	800	1.016	1.46	1.08	22	5.0	
12	740	1.015	1.34	0			
13	1600	1.010	1.49	0			Very cold; attended football game. Small amount of meat at night.
14	960	1.013	1.48	0			Meat.
15	670	1.023	1.52	0			Meat.
16	870	1.016	1.50	0			Meat.

creatine on isolated days, as has been the case in experiments previously reported on man.

Especial care was taken to make the data as reliable as possible. The creatine employed had been repeatedly recrystallized until snow-white and contained only a very small amount of creatinine, under one-quarter of 1 per cent. This is unimportant, since the conversion of creatine to creatinine was greatly in excess of this amount, as indicated by the increase in the excretion of creatinine.

The creatine was weighed with the water of crystallization, allowance being made for this increased weight, to avoid any possible conversion to creatinine. After being carefully dissolved in about 250 cc. of warm water, it was taken from one to one and one-half hours previous to the noon meal. This was believed to be a favorable time for rapid absorption.

TABLE II.

Experiments on M. F. S., November 3 to 18, 1913.

DAY	VOLUME OF URINE	SPECIFIC GRAVITY	CREATININE	DATA ON CREATINE: Excreted in urine	Excreted in urine	Taken by mouth	REMARKS ON DIET, ETC.
	cc.		gm.	gm.	per cent	gm.	
1	640		1.35	0			No meat, but meat on preceding day.
2	900		1.29	0			
3	640	1.020	1.29	0			
4	840	1.016	1.29	0			
5	600	1.020	1.29	0			
6	645	1.020	1.29	0			
7	1100	1.011	1.26	0.20	20	1.0	
8	1205	1.014	1.32	0.58	29	2.0	
9	1050	1.015	1.33	0.93	31	3.0	
10	520	1.029	1.35	1.42	37	4.0	
11	1100	1.013	1.37	1.97	39	5.0	
12	600	1.021	1.34	0			
13	1045	1.013	1.29	0			
14	1200	1.015	1.34	0			
15	610	1.026	1.51	0			Meat.
16	550	1.026	1.34	0			Meat.

Feeding experiments with creatine have been criticized on the ground that the creatine lost was destroyed in the intestine by bacterial action.[3] This criticism we do not believe to be valid for the reasons, (1) that the probable rate of absorption would not allow an appreciable loss in this way, and (2) that the upper portion of the intestine is comparatively sterile[4] under normal conditions.

[3] F. W. Twort and E. Mellanby: *Jour. Physiol.*, xliv, p. 43, 1912–13.
[4] W. J. MacNeal and A. F. Chace: *Arch. Int. Med.*, xii, p. 178, 1913.

The usual analytical methods were employed for the urine. In the instances where the volume was under 1 liter it was made up to this amount with distilled water. Care was taken in making the colorimetric readings to have the dilution so adjusted that the reading was close to 8 mm. The high results for creatinine on the first day of the control period compared with the succeeding five days are believed to be due to a lag in the excretion of exogenous creatinine, since this was the first day after the discontinuance of meat. The increase in the excretion of creatinine was slightly less in subject M. S. F. than in V. C. M., probably for the reason that in the former subject about one-third of the creatine reappeared in the urine, while in the latter only one-fifth was lost in this way.

DISCUSSION.

The results in the experiments here reported on two men are quite similar to those previously obtained on rabbits. We believe that the increase in the excretion of creatinine though small, 3 to 4 per cent, is slightly beyond the limits of experimental error under the conditions of our experiments. This represents a conversion of between 1.5 and 2 per cent of the creatine to creatinine, and is in harmony with the hypothesis previously presented by us, that administered creatine suffers the same fate as the creatine present in the body.

Folin and Denis,[5] in a paper which has appeared since the completion of the present experiments, found that the administration of creatine to cats results in its absorption by the muscles to a considerable extent, an observation in harmony with our own findings on rabbits; although in our experiments the muscle was not analyzed until one to four days after the administration of creatine had been discontinued. As might have been expected the actual retention was smaller than that noted by Folin and Denis. Folin and Denis state, however, that their experiments failed to show any creatinine formation from the administered creatine, although they noted a slight accumulation of creatinine in the blood and a slight diminution in the muscle. They suggest that the increased creatinine excretion noted by

[5] O. Folin and W. Denis: this *Journal*, xvii, p. 493, 1914.

other investigators may find possible explanation in the large influx of creatine into the muscle tending to force out the creatinine, although by what mechanism they do not explain.

Since creatine is slowly changed to creatinine even in pure solution and much more rapidly in autolyzing muscle tissue,[6] we believe the slight increase in the creatinine excretion above noted actually comes from the administered creatine.

[6] Myers and Fine: *Proc. Soc. Exper. Biol. and Med.*, xii, p. 41, 1914; also this *Journal*, xxi, 1915 (in press).

THE METABOLISM OF CREATINE AND CREATININE.

EIGHTH PAPER.

THE PRESENCE OF CREATININE IN MUSCLE.

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(Received for publication, April 24, 1915.)

In several papers[1] we have called attention to the presence of creatinine in muscle tissue, although our experimental data have never been presented. Since this is a topic about which there has been considerable controversy, and, further, since we have attached special significance to the creatinine of the muscle, it has been considered desirable to discuss separately our experiments dealing with this problem.[2]

The presence of creatinine in muscle was apparently a disputed question with the older investigators, and with the advent of the Folin colorimetric method, this topic again received attention. Dorner[3] and von Fürth and Schwarz[4] in experiments on striated muscle, and Saiki[5] in experiments on smooth muscle, all reported the presence of considerable amounts of creatinine—to the extent of one-third of the total creatinine. It seems quite apparent that the experiments of these workers were carried out without adequate precautions to prevent a conversion of creatine to creatinine and their data on this point may, therefore, be disregarded. In contrast to these observations are the experiments of many of the other workers who have considered this question. Grindley and Woods[6]

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[1] V. C. Myers and M. S. Fine: this *Journal*, xv, p. 304, 1913; xvi, p. 174, 1913–14; xvii, p. 65, 1914; Fine and Myers: *Proc. Soc. Exper. Biol. and Med.*, xi, p. 15, 1913.

[2] A discussion of the formation of creatinine from creatine may be found in a paper to appear in the next issue of this *Journal*.

[3] G. Dorner: *Ztschr. f. physiol. Chem.*, lii, p. 265, 1907.

[4] O. von Fürth and C. Schwarz: *Biochem. Ztschr.*, xxx, p. 418, 1911.

[5] T. Saiki: this *Journal*, iv, p. 483, 1908.

[6] H. S. Grindley and H. S. Woods: *ibid.*, ii, p. 309, 1906–07.

stated that they were unable to detect creatinine in chicken or beef flesh. Mendel and Leavenworth[7] likewise reported unsuccessful attempts to detect creatinine in embryonic muscular tissue, although it should be noted that only comparatively small amounts of creatine were found (0.03 per cent). Mellanby,[8] in his paper dealing with the creatine-creatinine problem in general, took up this question under the heading of "the alleged presence of creatinine in muscle." As the result of his work he concluded "that creatinine is never present in muscle in quantities capable of detection." His failure to detect creatinine in fresh muscle is not surprising, but it is difficult to account for his unsuccessful attempts in the case of autolyzed muscle where he must have been dealing with comparatively large amounts of creatinine. On the basis of these negative findings, however, Mellanby took occasion to criticize severely the work of Gottlieb and Stangassinger,[9] who reported the presence of creatinine in fresh as well as in autolyzing tissue. Other English workers who have investigated the presence of creatinine in muscle seem to have been influenced by the work of Mellanby. Brown and Cathcart[10] stated that they were inclined to agree with Mellanby that muscle contains no preformed creatinine, or at least mere traces of this substance. Paton[11] was unable to detect creatinine in duck muscle, but expressed some doubt in the case of rabbit muscle. Chisolm,[12] employing Mellanby's method for human muscle, stated that creatinine was always found to be absent. In a more recent communication Scaffidi[13] concluded that creatinine as a preformed chemical constituent is absent from the muscle tissue of the frog and dog and probably from muscle tissue in general. Shaffer appears to have been the first to appreciate adequately the probable amounts of creatinine present in muscle and other tissues, and the precautions necessary for its detection and estimation. In 1909 in a preliminary communication with Reinoso,[14] he suggested a method for the estimation of creatinine in dilute solutions and reported the presence of 1 to 6 mg. of creatinine in 100 gm. of dog muscle. To this creatinine he attached considerable metabolic significance. Quite recently he has made the experimental details of these interesting and important studies available.[15] In a single experiment

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[7] L. B. Mendel and C. S. Leavenworth: *Am. Jour. Physiol.*, xxi, p. 100, 1908.

[8] E. Mellanby: *Jour. Physiol.*, xxxvi, p. 455, 1907–08.

[9] R. Gottlieb and R. Stangassinger: *Ztschr. f. physiol. Chem.*, lii, p. 1, 1907.

[10] T. G. Brown and E. P. Cathcart: *Biochem. Jour.*, iv, p. 425, 1909.

[11] D. N. Paton: *Jour. Physiol.*, xxxix, p. 489, 1909–10.

[12] R. A. Chisolm: *Biochem. Jour.*, vi, p. 244, 1911–12.

[13] V. Scaffidi: *Biochem. Ztschr.*, 1, p. 402, 1913.

[14] P. A. Shaffer and E. A. Reinoso: this *Journal*, vii, pp. xiii and xxx, 1910.

[15] Shaffer: *ibid.*, xviii, p. 525, 1914.

carried out in connection with other work in 1912, Folin and Denis[16] reported the presence of a trace of creatinine in normal cat muscle.

Our studies on creatine and creatinine likewise led us to an investigation of this problem, and, independently of the work of Shaffer, we developed a method which we believe satisfactorily answered the question regarding the presence of creatinine in muscle.[17] Since our method was described, Folin[18] has suggested a very simple procedure for the estimation of the preformed creatinine in tissues in general. Below are tabulated results obtained by our original method as well as a few additional figures obtained by Folin's new method.

Creatinine content of muscle.

SOURCE OF MATERIAL	DATE	CREATININE CONTENT OF FRESH MUSCLE	REMARKS
Authors' method.			
		mg. per 100 gm.	
Rabbit 65	June 9, 1913	6.4	
Rabbit 66	June 13, 1913	6.4	
Rabbit 67	June 19, 1913	8.8	
Rabbit 69	June 27, 1913	9.2	
Rabbit 73*	Aug. 4, 1913	7.0	Creatine injection.
Rabbit 74*	Aug. 4, 1913	9.0	Creatinine injection.
Rabbit 75	Sept. 17, 1913	a 3.7	Clarified by colloidal iron.
		b 3.4	Clarified by precipitated ferric hydroxide.
		c 3.8	Clarified by alumina cream.
Rabbit 77†	Sept. 30, 1913	6.8	
Rabbit 82	Dec. 6, 1913	7.1	Animals given phlorhizin.
Rabbit 83	Dec. 10, 1913	5.0	
Rabbit 84	Feb. 14, 1914	12.9	Animals died after feeding with large amounts of "glidin," albuminuria, nephritis (?).
Rabbit 85	Feb. 14, 1914	14.5	
Cat 17	Nov. 10, 1913	4.8	
Dog 6	Nov. 3, 1913	5.0	
Human 6	Jan. 30, 1914	7.0	Diabetes, glomerular nephritis.
Human 7	Feb. 3, 1914	2.9	Pneumonia.

* Myers and Fine: this *Journal*, xvi, p. 174, 1913.
† Fine and Myers: *Proc. Soc. Exper. Biol. and Med.*, xi, p. 15, 1913.

[16] O. Folin and W. Denis: *ibid.*, xii, p. 151, 1912.
[17] Myers and Fine: *ibid.*, xvii, p. 65, 1914.
[18] Folin: *ibid.*, xvii, p. 475, 1914.

Creatinine content of muscle—Continued.

SOURCE OF MATERIAL	DATE	CREATININE CONTENT OF FRESH MUSCLE	REMARKS
		Folin's method.	
Rabbit 88	Oct. 19, 1914	4.5	
Rat 19	June 12, 1914	4.0	
Rat 20	June 12, 1914	8.0	
Cat 18	Apr. 7, 1915	5.9	
Hen 1	Feb. 26, 1915	1.6	Leg muscle.
		5.3	Breast muscle.
Hen 2	Mar. 2, 1915	1.9	Leg muscle.
		5.0	Breast muscle.
Human 9	June 26, 1914	5.7	Amputation of leg for sarcoma.
Human 11	Mar. 3, 1915	2.6	Amputation of leg for gangrene.
Human 10§	Jan. 19, 1915	6.8	Pectoral muscle } interstitial nephritis, uremia.
		6.8	Heart muscle } interstitial nephritis, uremia.
Human 12	Mar. 23, 1915	18.1	Psoas muscle } interstitial nephritis, uremia.
		15.5	Heart muscle } interstitial nephritis, uremia.
Human 13	Apr. 12, 1915	4.0	Cholelithiasis.
Uterus 2	Apr. 21, 1915	1.2	Fibroid.
Uterus 3	Apr. 22, 1915	2.2	
Thyroid	Apr. 5, 1915	1.1	Exophthalmic goitre.

§ Myers and Fine: this *Journal*, xx, p. 397, 1915.

Essentially the same range of figures is found with the two methods employed, the values likewise being comparable with those obtained by Shaffer. Our own method has not been directly compared with that of Folin, since we have satisfied ourselves that both methods give comparatively accurate results. Obviously where such small quantities of material are considered, it is difficult to avoid slight sources of error, amounting in some cases possibly to 5 to 15 per cent, but this can in no way invalidate the conclusions to be drawn. With our method an appreciable length of time is required for evaporation with an air current, while with the Folin method, complete extraction of the creatinine is obviously difficult. In our work with the Folin method we have preferred after grinding the muscle with sand, to extract with water instead of saturated picric acid, previous to the final addition of the dry picric acid. On account of its greater simplicity Folin's method for the determination of the creatinine is to be preferred to our own.

DISCUSSION.

Since creatinine is eliminated in the urine in considerable quantity, it obviously must be present in the tissues, as indeed Folin[19] argued ten years ago. It is evident from the data tabulated above that preformed creatinine is always present in muscle tissue. Furthermore, as pointed out by Shaffer[20] and noted by Folin and Denis,[21] the quantity of creatinine present in the muscle is much greater than that of the blood,[22] liver, or in fact any other tissue. The following analyses from the tissue of a cat illustrate this point (figures calculated in mg. per 100 grams of tissue): muscle, 5.9; spleen, 4.0; smooth muscle (stomach), 3.2; liver, 2.8; and blood, 2.4. The fact that the greater portion of the preformed creatinine present in the body is found in the muscle strongly suggests that this is the chief creatinine-forming tissue. Apparently the blood is unable to remove the creatinine from the muscle quite as rapidly as it is formed. The objection to this argument may be offered that the muscle tissue has some special affinity for the creatinine as it has in a far greater degree for creatine. That this is not the case seems evident from the fact that in severe uremia in the human subject and in rabbits with double nephrectomy, both of which are accompanied by a marked retention of creatinine, the concentration of creatinine in the blood may rise considerably above that of the muscle tissue.[23] These observations all strongly support the view long held by Shaffer[24] and ourselves[25] that he urinary creatinine finds its origin chiefly in the muscle tissue.

[19] Folin: *Am. Jour. Physiol.*, xiii, p. 84, 1905.

[20] Shaffer: *loc. cit.*

[21] Folin and Denis: *ibid.*, xvii, p. 501, 1914.

[22] From the blood analyses reported by Folin and Denis (this *Journal*, xvii, p. 487, 1914) and Myers and Fine (*ibid.*, xx, p. 391, 1915), it is evident that normally the creatinine of the blood amounts to 1 to 2 mg. per 100 cc., or about one-fourth that found in the muscle.

[23] The experiments containing these data will be described in another paper.

[24] Shaffer: *Am. Jour. Physiol.*, xxiii, p. 4, 1908-09.

[25] Myers: *Am. Jour. Med. Sc.*, cxxxix, p. 256, 1910. Myers and Fine: this *Journal*, xiv, p. 24, 1913.

THE METABOLISM OF CREATINE AND CREATININE.

NINTH PAPER.

THE CREATINE CONTENT OF THE MUSCLE OF RATS FED ON ISOLATED PROTEINS.

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(Received for publication, April 24, 1915.)

The possible origin of creatine from arginine has long been a subject of discussion,[1] although no direct evidence has as yet been presented which conclusively proves arginine to be the mother substance of creatine. Through the courtesy of Doctor Osborne and Professor Mendel we have had the opportunity of determining the creatine concentration of the muscle of rats, some of which had been fed on a protein rich in arginine, others on a protein comparatively poor in its yield of arginine. In most of the experiments in question the sole protein was either edestin or casein, the former yielding 14 per cent arginine, the latter 4 per cent.[2]

From the data tabulated below it is apparent that the concentration of the muscle creatine of all the animals is quite uniform, although slightly lower values are noted in the casein series, when compared with the edestin and control rats. We hesitate at the present time, however, to draw definite conclusions from these slight differences, since they may possibly be attributed to variations in the nutritive conditions of the

[1] See C. J. C. van Hoogenhuyze and H. Verploegh: *Ztschr. f. physiol. Chem.*, xlvi, p. 433, 1905. M. Jaffé: *ibid.*, xlviii, p. 464, 1906. K. Inouye: *ibid.*, lxxxi, p. 71, 1912. See also O. Riesser: *ibid.*, lxxxvi, p. 415, 1915, and A. Palladin and L. Wallenburger: *Compt. rend. Soc. de biol.*, lxxviii, p. 111, 1915.

[2] T. B. Osborne: *The Vegetable Proteins*, New York, 1909, p. 59.

two sets of animals,[3] and furthermore are scarcely beyond the limits of accuracy with the colorimetric method.

Creatine content of the muscle of rats fed on isolated proteins.

OSBORNE AND MENDEL'S SERIAL NO. SEX	AGE	WEIGHT	DIET (PROTEIN)	MUSCLE ANALYSES	
				Water	Creatine
	days	*gm.*		*per cent*	*per cent*
1592♂	375	305	Casein	73.7	0.453
2030♂	139	246	"	76.3	0.454
2098♂	114	211	"	73.3	0.453
2005♂	149	206	"	71.2	0.464
1636♀	372	198	"	72.3	0.453
1973♂	169	194	"	74.6	0.458
2106♂	115	175	"	73.6	0.470
1919♀	192	161	"	72.7	0.465
1988♀	186	160	"	72.3	0.453
2134♂	80	157	"	74.1	0.453
2145♀	79	124	"	74.8	0.464
1615♂	377	303	Edestin	74.4	0.476
1893♂	217	295	"	73.9	0.470
1948♂	191	260	"	74.2	0.467
1593♂	391	255	"	75.0	0.476
1920♀	200	187	"	74.9	0.470
1927♀	190	150	"	72.7	0.468
2110♀	110	128	"	74.6	0.464
1346♂	466	247	Lactalbumin	74.2	0.464
2115♂	95	173	"	74.1	0.458
1545♀	450	213	Mixed-control	75.3	0.476
1939♀	236	160	Mixed-control	76.0	0.475

In an earlier communication[4] we called attention to the very uniform results that were obtained for the creatine content of the muscle of certain animals, particularly the rabbit, and suggested that this might ultimately be found to be the underlying factor in the constancy in the excretion of creatinine. Our results, as we then pointed out, were in accord with the findings

[3] For discussion of this phase of the subject and records of a number of the rats in the present series, see T. B. Osborne and L. B. Mendel: this *Journal*, xx, p. 351, 1915.

[4] V. C. Myers and M. S. Fine: *ibid.*, xiv, p. 9, 1913.

of Dorner[5] and Mellanby[6] for this animal, and have been confirmed by the experiments of Riesser, and Palladin and Wallenburger.[7] The more recent observations of Baumann[8] on two animals in which the analyses were checked by different methods also harmonize with our results.

In a still more recent communication Folin and Buckman[9] have taken exception to this view. From their results they conclude that "the variations found" (in the creatine content of muscle) "appear to be too large to permit the use of average figures in calculations as to the alleged relationship between the creatinine elimination and the total amount of creatine in the tissues." In their paper they criticize the extraction method for the determination of creatine on the basis of its representing minimal values. This supposition scarcely finds support in our published data when one notes the comparatively uniform results which we obtained. If carefully carried out, the loss in the extraction method is certainly less than 1 per cent, or within the limits of accuracy with the colorimetric method. In our original experiments[10] constant values were found for the creatine content of the muscle of the rabbit and dog, but not for the cat. We suggested at that time that the variations in the cat muscle might find explanation on the basis of differences in diet and general nutrition. With regard to our rabbits, it may be noted here that they were fed upon a comparatively uniform diet. The dogs were miscellaneous animals killed in the laboratory for other purposes shortly after being received. No particular comments can be made with regard to their diet, except to mention that they probably had not been overfed. In this case the analyses were made at different times and entirely independently of each other.

[5] G. Dorner: *Ztschr. f. physiol. Chem.*, lii, p. 259, 1907.

[6] E. Mellanby: *Jour. Physiol.*, xxxvi, p. 447, 1907–08.

[7] Riesser (*loc. cit.*, p. 444) found in his six control rabbits, 0.528, 0.521, 0.521, 0.521, 0.521, and 0.521 per cent of creatine in the muscle; while Palladin and Wallenburger (*loc. cit.*) found for the muscle of their eight normal rabbits the following figures: 0.522, 0.528, 0.517, 0.522, 0.545, 0.539, 0.522 and 0.526 per cent creatine.

[8] L. Baumann: this *Journal*, xvii, p. 15, 1914.

[9] O. Folin and T. E. Buckman: *ibid.*, xvii, p. 483, 1914.

[10] Myers and Fine: *loc. cit.*

We do not intend to imply from the above remarks that the muscle creatine of the rabbit, dog, and rat may not vary from the rather narrow limits of our reported analyses. It is evident from our starvation experiments[11] on rabbits that wide variations may be encountered in undernutrition, and we may further note that in our first series of experiments 0.602 per cent creatine was found in the muscle of Rabbit 18, and 0.427 per cent in Rabbit 22. These figures were not included in our table because the rabbits were unhealthy animals.

It seems quite evident from the data at hand[12] that muscle tissue is able to absorb appreciable amounts of creatine. Since the cat is a carnivorous animal, and obviously consumes varying amounts of creatine, this would appear to afford an explanation for the variations in the concentration of muscle creatine. The diets of our rabbits and rats, on the other hand, were creatine- and creatinine-free in the usual sense, and on this account it would not be unreasonable to expect a fairly uniform content of creatine in the muscle tissue in these animals under normal conditions. Such we believe to be the case.

Since Folin and Buckman have questioned the accuracy of the extraction method for the estimation of creatine in muscle tissue, the procedure employed in the present analyses will be briefly described: 10 to 20 gram samples of the finely ground muscle, which had been preserved in alcohol, were treated with about 5 volumes of 0.01 N acetic acid in casseroles and heated on the water bath until protein coagulation was complete. The material was then filtered through a folded hardened filter paper, the coagulum returned to the casserole and extracted with about 200 cc. of hot water, filtered, and the process repeated a third time. The combined filtrates were then concentrated in an evaporating dish on the water bath and finally made up to a volume such that 100 cc. represented 10 grams of muscle. A fourth extraction was always made but rarely gave, after evaporation and on hydrolysis, more than a slight, if any, reaction for creatinine. It is not believed that the loss by this method as here carried out was ever over one-half of 1 per cent. The creatine was transformed

[11] Myers and Fine: this *Journal*, xv, p. 283, 1913.

[12] Myers and Fine: *ibid.*, xvi, p. 169, 1913–14. O. Folin and W. Denis: *ibid.*, xvii, p. 493, 1914.

to creatinine and estimated in the manner previouslyd escribed,[13] dilution being made to 200 cc. for the colorimetric reading, whereby the readings all fell between 7.9 and 8.3 mm. on the scale of the Duboscq colorimeter, the latest model being employed. Since a half dozen extractions may be simultaneously carried out, the method is not especially time-consuming. With human tissue the same extract has been employed to advantage for the estimation of uric acid, urea, and chlorides.

In some instances we have employed Folin's[14] new method as well as the extraction method, generally with good agreement between the two procedures. When discrepancies have occurred, however, the low results have been with the Folin method.

The assertion of Folin and Buckman that all determinations made on the basis of the extraction method represent minimum values, is not supported by our analyses nor by those of Baumann. Folin and Buckman have offered no evidence in support of the greater accuracy of their method; and in view of the widely varying figures yielded by it, their criticism of the uniform results obtained by various workers does not appear to us well founded.

CONCLUSIONS.

The creatine content of rat muscle varies within comparatively narrow limits, at least under the dietary conditions of the animals here considered. The concentration of the muscle creatine does not appear to be markedly influenced by the feeding of proteins having a high or low content of arginine, although it should be noted that on the casein diet (poor in arginine) the content of creatine appears to be slightly lower than the control and edestin rats (about 2.5 per cent below that of the edestin animals).

[13] Myers and Fine: *ibid.*, xvii, p. 67, 1914.
[14] Folin: *ibid.*, xvii, p. 480, 1914.

CHANGES IN THE FAT CONTENT OF FECES PRESERVED BY FREEZING WITHOUT THE ADDITION OF A PRESERVATIVE.

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(Received for publication, April 20, 1915.)

In connection with some experiments concerning the relative degree of digestion and utilization of lard and hydrogenated vegetable oils,[1] opportunity was offered for the study of the effect of freezing on the fat of feces to which no preservative had been added. Thymol is frequently used for the preservation of feces, but since fat was to be determined in the preserved material by extraction with ether, it was thought not advisable to use this preservative; and as there were some difficulties connected with the use of any other substance, it was decided to try the effect of freezing alone. The possibility that simple freezing would be sufficient was suggested by work of Howe, Rutherford, and Hawk,[2] who showed that freezing was a suitable method for preserving thymolized feces in which nitrogen was to be determined.

Feces were collected from two subjects who were on diets of uniform fat content during two periods of eight days each. The two periods were separated by an interval of three days. Subject S received 92.0 grams of fat per day throughout the first period, and 94.0 grams of fat per day during the second period; whereas Subject M received each day of the two periods 94.4 grams and 96.6 grams respectively. Each day's stool was collected in a weighed, flat, porcelain plate and was thoroughly mixed immediately after defecation. The mixing was done with

[1] Smith, Miller, and Hawk: unpublished.

[2] P. E. Howe, T. A. Rutherford, and P. B. Hawk: *Jour. Am. Chem. Soc.*, xxxii, p. 1683, 1910.

the aid of a weighed steel spatula. After mixing, the total weight of the plate, spatula, and stool was obtained on a torsion balance, and the weight of the stool thus obtained by difference. One-tenth of the stool was at once placed in a weighed friction-top can, as part of the composite for the period, and quickly frozen in a brine tank at $-12°$ C. The composite cans were removed from the freezing tank each day for the addition of the daily portions. In this way some slight thawing occurred during the time the cans were out of the brine tank. This thawing was relatively small as the size of the composite increased. Another part of the day's stool was quickly transferred to a glass-stoppered weighing bottle. This portion of each day's stool was at once analyzed for total fat, fatty acid, and neutral fat. The weighing bottle contained, in addition to the feces, a glass rod, the end of which was flattened and bent in the form of a hoe. This glass hoe was used in transferring the feces from the weighing bottle to the cylinder in which the material was extracted.

Some time after the end of the entire experiment (about five weeks after the first sample was taken) the composite cans, containing one-tenth of each day's stool, were removed from the brine tank, thawed, thoroughly mixed, and weighed. The weight of the can was subtracted in order to obtain the weight of the composite feces. Ten times this weight, then, represented the total stool of the period. After mixing and weighing, a sample was transferred to a weighing bottle and immediately analyzed for total fat, fatty acid, and neutral fat. The values obtained from the composites were calculated to the total feces of the periods. These were compared with the respective eliminations calculated to the total feces from the analysis of the daily stools. The comparisons of the total fat, fatty acid, and neutral fat in the composite feces of the four periods, with total fat, fatty acid, and neutral fat in the daily feces for the same periods, are shown in Tables I, II, III, and IV. The composite feces were *frozen*, whereas the daily samples were analyzed in the *fresh* condition.

The Saxon method[3] was used for the determination of the total fat, fatty acid, and neutral fat of the feces. The method proved to be speedy, convenient, and accurate, and seems to overcome most of the disadvantages of simple extraction methods as sum-

[3] G. J. Saxon: this *Journal*, xvii, p. 99, 1914.

marized by Gephart and Csonka,[4] who apparently failed to consider the Saxon method.

TABLE I.

Subject S. First period.

STOOL		WEIGHT	TOTAL FAT	FATTY ACID	NEUTRAL FAT
		gm.	*gm.*	*gm.*	*gm.*
Daily (unfrozen)	1	42.0	1.39	0.28	1.11
	2	90.6	3.47	1.33	2.14
	3	139.8	4.18	1.83	2.35
	4	245.3	6.00	2.84	3.16
	5	189.5	3.77	1.59	2.18
	6	142.2	3.12	1.51	1.61
	7	179.3	4.54	1.72	2.82
	8	103.4	2.41	1.00	1.41
	9	99.3	2.92	.33	2.59
	10	34.8	1.81	.50	1.31
Totals		1266.2	33.61	12.93	20.68
Composite (frozen)			27.20	14.52	12.68
Differences			−6.41	+1.59	−8.00

TABLE II.

Subject S. Second period.

STOOL		WEIGHT	TOTAL FAT	FATTY ACID	NEUTRAL FAT
		gm.	*gm.*	*gm.*	*gm.*
Daily (unfrozen)	1	254.1	3.64	1.42	2.22
	2	231.7	4.67	2.04	2.63
	3	107.7	3.32	1.19	2.13
	4	377.5	10.15	4.19	5.96
	5	79.0	3.22	1.42	1.80
	6	254.1	8.37	3.43	4.94
	7	114.0	3.30	1.34	1.96
	8	178.2	5.16	1.82	3.34
	9	87.8	3.41	0.49	2.92
Totals		1684.1	45.24	17.34	27.90
Composite (frozen)			41.47	24.75	16.72
Differences			−3.77	+7.41	−11.18

[4] F. C. Gephart and F. A. Csonka: *ibid.*, xix, p. 521, 1914.

TABLE III.

Subject M. First period.

STOOL		WEIGHT	TOTAL FAT	FATTY ACID	NEUTRAL FAT
		gm.	*gm.*	*gm.*	*gm.*
Daily (unfrozen)	1	172.2	4.00	1.39	2.61
	2	86.0	2.31	0.95	1.36
	3	191.0	3.82	1.99	1.83
	4	111.8	3.44	1.64	1.80
	5	220.9	5.58	3.08	2.50
	6	190.7	6.43	1.58	4.85
	7	448.2	8.15	3.58	4.57
	8	346.9	7.95	2.36	5.59
	9	95.0	3.40	1.36	2.04
Totals.................		1862.7	45.08	17.93	27.15
Composite (frozen)....			38.84	27.72	11.12
Differences............			−6.24	+9.79	−16.03

TABLE IV.

Subject M. Second period.

STOOL		WEIGHT	TOTAL FAT	FATTY ACID	NEUTRAL FAT
		gm.	*gm.*	*gm.*	*gm.*
Daily (unfrozen)	1	92.9	3.95	1.96	1.99
	2	179.3	7.81	1.89	5.92
	3	280.5	9.05	4.49	4.56
	4	189.5	7.03	3.45	3.58
	5	253.2	6.76	3.70	3.06
	6	421.7	8.52	4.47	4.05
	7	387.0	6.65	3.60	3.05
	8	425.5	6.51	3.57	2.94
Totals.................		2229.6	56.28	27.13	29.15
Composite (frozen)			50.49	34.78	15.71
Differences............			−5.79	+7.65	−13.44

Gephart and Csonka state that: "The simple extraction methods can be discarded after a few considerations, the most important of which are the following:

(a) The material to be extracted must be dried and finely powdered or a very slow wet extraction must be resorted to, in which latter case the

extract obtained will not be a true extract of the solvent employed but will contain some water-soluble material.

(b) A large proportion of the fatty acid complex in feces is in the form of soaps, which are either wholly or in part insoluble in the solvent.

(c) The solvent extracts materials other than fat, such as cholesterin, coloring matter, etc., which may constitute 50 per cent of the total extract.

(d) Solvents do not come in intimate contact with the material; after the extraction has proceeded for a time there is always more or less 'packing.' "

All of these objections, with the exception of (c), are overcome by the Saxon method, and in much work in which uniform diets are fed the error due to removal of material other than fat is constant and hence not of moment. The Saxon method is carried out with *moist* material, yet it is *not slow;* and since there is a subsequent purification of the first extract by means of petroleum ether, the objection of extracting *water-soluble material* does not hold. The difficulty due to the non-solubility of soaps in ether is avoided by first breaking up the soaps by means of hydrochloric acid. This affords a ready means of determining, on a separate sample without adding hydrochloric acid, the amount of fatty acid originally present in the form of soaps, which point is of considerable importance in certain instances of intestinal disturbance.[5] The objection on the ground of "packing" is obviously not applicable to the Saxon method, in which the three five minute periods of shaking serve to keep the material finely divided and in intimate contact with the solvent.

From the data given in the four tables it will be seen that there was in all cases a decrease in the total fat of the frozen feces. It will be noted also that in the feces which had been frozen for the longer time (Tables I and III) there was a greater loss of total fat, 6.41 grams and 6.24 grams respectively, than was the case with the feces frozen for the shorter time (Tables II and IV), in which the loss was only 3.77 grams and 5.79 grams respectively. The losses of total fat do not appear to be uniform, however, for feces from different individuals. The destruction of fat seems to be roughly proportional to the length of time the material had been kept, and it is probable that this destruction was caused by bacterial action, although no attempt was made in this experiment to determine this point.

[5] P. J. Cammidge: *The Faeces of Adults and Children*, Bristol, 1914, p. 266.

There is to be noted in all cases, in addition to the destruction of total fat, a marked decrease in the amount of neutral fat, and considerable increase in the amount of fatty acid, in the frozen feces as compared with the corresponding values in the fresh feces. There seems to be no direct relation between the increase in fatty acid and the length of time the feces were frozen. The increases of fatty acid in the feces of the first periods (frozen for the longer time) were 1.59 grams and 9.79 grams respectively (Tables I and III), while the increases of fatty acid in the feces of the second periods (frozen for the shorter time) were 7.41 grams and 7.65 grams respectively (Tables II and IV). It is thus seen that the losses in neutral fat (8.00, 11.18, 16.03, and 13.44 grams respectively) were due partly to the actual destruction of fat, and partly to lipolytic conversion of the neutral fat into fatty acids. Furthermore, as there seems to be no relation between the amount of fat actually destroyed and the amount of fatty acid formed, it may be that the splitting of the fat is not a necessary preliminary to its destruction. There is apparently little uniformity in the amounts of fatty acid formed in the different samples, which indicates a variation in the amount or activity of lipase present. No determinations were made of this enzyme, however. In connection with the lipolysis in frozen feces, which were kept at $-12°$ C., the work of Pennington and Hepburn[6] and of Pennington, Hepburn, and Connolly[7] is of interest. They were able to show that the lipase of chicken fat was inhibited in its action at low temperatures ($-12°$ C. was the lowest used), but that there was not a complete arrest of activity. Richardson[8] has shown, also, that pancreatic lipase (hog) retains some activity at freezing temperatures for two or three months. From the fact that there is a change of fat into fatty acid in feces kept at $-12°$ C., it is evident that pancreatic or intestinal lipase in feces must be at least slightly active at that low temperature.

[6] M. E. Pennington and J. S. Hepburn: *Jour. Am. Chem Soc.*, xxxiv, p. 210, 1912.

[7] Pennington, Hepburn, and E. L. Connolly: this *Journal*, xvii, p. xliv, 1914.

[8] W. D. Richardson: Premier Congrès International du Froid, Rapports et Communications, ii, p. 315, 1908.

We wish to take this opportunity of expressing our thanks to Dr. Gordon J. Saxon for helpful suggestions in regard to the details of the fat method.

CONCLUSIONS.

1. The fat of feces kept at a temperature of $-12°$ C. undergoes both hydrolysis (shown by the increase in fatty acid) and actual destruction (shown by the decrease in total fat). No attempt was made to determine the exact cause of the decrease in total fat.

2. Simple freezing without the addition of a preservative is not applicable to the preservation of feces in which fat is to be determined.

3. The Saxon method for the determination of fat in moist feces avoids most of the objections to which other simple extraction methods are subject, and has the added advantage of allowing for the differentiation between neutral fat and fatty acids (free and combined as soaps).

THE QUATERNARY SALTS OF HEXAMETHYLENE-TETRAMINE.

V. MONOHALOGENACETYL DERIVATIVES OF AMINOALCOHOLS AND THE HEXAMETHYLENETETRAMINIUM SALTS DERIVED THEREFROM.[1]

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The only reference to be found in the literature to the halogenacetyl derivatives of the aminoalcohols or alcamines is that contained in the work of Einhorn and his collaborators.[2] In the communications of these authors the halogenacetyl derivatives of the simplest alcamine, aminomethanol, are described, and along with these the quaternary salts obtained by combining them with hexamethylenetetramine. The present authors have had occasion to work somewhat extensively in this field and have made the results of these investigations the subject of the present communication.

In the biological studies with which the chemical work was associated, the results obtained at the time with certain members of this class of hexamethylenetetramine quaternary salts were such as to demand a detailed study of the field. It had been hoped at first that by concentrating our attention on the simplest member of the series, chloroacetylaminomethanol, in the way of acylating the hydroxyl group with a great variety of acids, a uniform series of substances would be obtained for biological study.

The acylation of the hydroxyl group to form chloroacetylaminomethyl esters was readily accomplished by the use of the pyridine method. In attempting, however, to combine these esters with

[1] For the preceding papers of this series, see this *Journal*, xx, pp. 659 and 685; xxi, pp. 103 and 145.

[2] A. Einhorn and collaborators: *Ann. d. chem.*, cccxliii, p. 207, 1905; ccclxi, p. 113, 1908.

hexamethylenetetramine, although addition occurred, we found it impossible to isolate the product sought. In each case the product obtained was the quaternary salt of the base with chloroacetylaminomethanol. It is possible that moisture was responsible for the saponification of the quaternary ester salt which was first formed.

By turning to the higher aminoalcohols the results obtained were more promising. Both the chloroacetylaminoalcohols and the esters derived from them reacted smoothly with hexamethylenetetramine to form stable salts. With chloroacetylaminoethanol, in particular, a large number of esters were prepared and combined with hexamethylenetetramine, forming a uniform series of compounds suited for the biological work. The chloroacetylaminoalcohols were prepared from the aminoalcohols and chloroacetyl chloride by the Schotten-Baumann method, and, in the case of the aliphatic compounds, were purified by distillation in high vacuum. The esters of chloroacetylaminoethanol were prepared by the acylation of this substance with the acid chloride in pyridine solution, and in some cases, though less advantageously, by the chloroacetylation of the aminoethyl esters obtained by Gabriel's method.[3]

This last procedure was also attempted with the esters of γ-aminopropanol and aminoisopropylalcohol, but the chloroacetylation by the Schotten-Baumann method was found to be uncertain. The aminopropyl esters obtained by the rearrangement of the bromopropylamides according to Gabriel's method were isolated as the hydrobromides. In attempts to chloroacetylate these with chloroacetyl chloride and alkali, the aminopropyl ester liberated by the alkali as free base rapidly rearranged to the oxypropylamide, and the product obtained was either a mixture of the chloroacetylaminopropyl ester and the chloroacetate of oxypropylamide or the latter alone. In the case of the aminoisopropyl ester of p-nitrobenzoic acid the rapidity of this rearrangement was very striking. The most suitable method for obtaining the chloroacetylaminopropyl esters, provided the parent aminoalcohols are accessible, is by direct acylation of the chloroacetylaminoalcohols.

[3] S. Gabriel and P. Heymann: *Ber. d. deutsch. chem. Gesellsch.*, xxiii, p. 2493, 1890.

The work was extended to include the derivatives of secondary aminoalcohols with the nitrogen substituted both by alkyl and aryl groups. The chloroacetyl derivatives of this type either did not react with hexamethylenetetramine or reacted so slowly that only in one or two cases was it possible to isolate a crystalline salt. Although the bromo- and iodo-derivatives reacted more rapidly, we were unable to isolate the products formed in crystalline condition. The bromo- and iodo-derivatives themselves proved to be substances difficult to obtain crystalline.

EXPERIMENTAL.

As in the preceding papers of this series, the hexamethylenetetraminium salts were prepared, unless otherwise stated, by boiling equimolecular amounts of the components in a small volume of dry, alcohol-free chloroform. With one or two exceptions, which are noted as they occur, the hexamethylenetetraminium salts described in this communication are readily soluble in water. We wish also to reiterate here what was stated in the first paper, that the melting points or rather decomposition points of these quaternary salts depend greatly upon the mode of heating and that for this reason the determination of the corrected melting points was useless.

Unless otherwise stated, the following method was used for the preparation of the esters of the chloroacetylaminoalcohols described below. This is given here in order to avoid unnecessary repetition. The chloroacetylaminoalcohol was dissolved in five parts by weight of pyridine, cooled in a freezing mixture, and carefully treated with a solution of 1.25 mols. of the acid chloride in a little dry, alcohol-free ether or chloroform. After standing for fifteen to thirty minutes at room temperature, the mixture was poured into iced 25 per cent sulphuric acid. If the reaction mixture is allowed to stand too long the yields are reduced, apparently owing to the formation of soluble pyridinium salts. The esters generally separated as oils which rapidly solidified, in some cases only after the removal of the ether or chloroform by means of a current of air. The crude products were thoroughly washed first with dilute sulphuric acid, then with 5 per cent sodium carbonate solution, and finally with water. Variations from the above procedure are denoted below as they occur.

Halogen in the intermediate products was determined by the Volhard method after hydrolysis with 10 per cent alcoholic sodium hydroxide.

(1) Oxymethylchloroacetamide and derivatives.

Oxymethylchloroacetamide and hexamethylenetetramine. This substance, already obtained by Einhorn and Göttler,[4] was more conveniently prepared by us as follows: Equimolecular amounts of the components were boiled for one and one-half to two hours in about ten parts of acetone. The precipitated salt was filtered off and washed well with dry chloroform. An additional amount was obtained by continued boiling of the filtrate from the first crop. As prepared in this way, the salt melts with decomposition at 154–5° and is not hygroscopic, as stated by the above authors. Yield: almost quantitative.

0.2435 gm. of substance required 9.13 $AgNO_3$ Solution I.[5]
Calculated for $C_9H_{18}O_2N_5Cl$: Cl = 13.45 per cent.
Found: Cl = 13.20 per cent.

Chloroacetylaminomethyl benzoate, $ClCH_2CONHCH_2OOCC_6H_5$. The ester, obtained by the method outlined above, was recrystallized twice from absolute alcohol, then once from toluene. It forms prismatic rods which melt at 73–3.5° (corrected) with slight preliminary softening. It is very soluble in chloroform, less easily in ether and benzene.

0.2335 gm. of substance (Kjeldahl) required 10.2 cc. $\frac{N}{10}$ HCl.
0.1468 gm. of substance required 12.09 cc. $AgNO_3$ Solution II.[6]
Calculated for $C_{10}H_{10}O_3NCl$: N = 6.16 per cent; Cl = 15.58 per cent.
Found: N = 6.12 per cent; Cl = 15.32 per cent.

Attempts to prepare the hexamethylenetetraminium salt of the compound by the usual methods resulted only in the isolation of the impure quaternary salt of oxymethylchloroacetamide.

Chloroacetylaminomethyl anisate (*chloroacetylaminomethyl p-methoxybenzoate*). The crude ester, obtained in practically quantitative yield, was recrystallized first from absolute alcohol, then

[4] A. Einhorn and M. Göttler: *Ann. d. chem.*, ccclxi, p. 150, 1908.
[5] 1 cc. = 0.00352 gm. Cl; 0.00793 gm. Br; 0.01259 gm. I.
[6] 1 cc. = 0.00186 gm. Cl; 0.004192 gm. Br; 0.006657 gm. I.

benzene containing a little toluene to prevent its freezing in the ice box. It forms large rosettes of delicate hairs which melt at 106–7° (corrected) with slight preliminary sintering. It is somewhat soluble in ether, very easily in chloroform.

0.2089 gm. of substance (Kjeldahl) required 8.25 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{11}H_{12}O_4NCl$: N = 5.44 per cent.
Found: N = 5.53 per cent.

Attempts to prepare the quaternary salt by the usual methods failed as in the case of the benzoate.

(2) Derivatives of chloroacetylaminoethanol.

Chloroacetylaminoethanol, $ClCH_2CONHCH_2CH_2OH$. 25 grams of redistilled aminoethyl alcohol (Kahlbaum) were dissolved in 100 cc. of water and treated with 225 cc. of two-normal sodium hydroxide solution. After cooling in a freezing mixture, 50 grams of chloroacetyl chloride were added drop by drop, with vigorous shaking and continued cooling. At the end, an excess of 10 per cent sodium hydroxide solution was added until the reaction was definitely alkaline, after which the mixture was neutralized with hydrochloric acid and concentrated as far as possible *in vacuo.* The residue was taken up in dry acetone and filtered from the sodium chloride. The filtrate was again concentrated and the treatment with dry acetone repeated. After again concentrating the filtrate the residue was fractionated *in vacuo.* The chloroacetylaminoethanol boils at 141–5° (corrected, bath at 180–90°) at 0.25–0.35 mm. and solidifies in a freezing mixture, melting again unsharply as the temperature is raised. Yield: 75 per cent of the theory. The compound is readily soluble in water, alcohol, and acetone, difficultly in chloroform.

0.2550 gm. of substance (Kjeldahl) required 18.01 cc. $\frac{N}{10}$ HCl.
0.1452 gm. of substance required 19.98 cc. $AgNO_3$ Solution II.
Calculated for $C_4H_8O_2NCl$: N = 10.18 per cent; Cl = 25.78 per cent.
Found: N = 9.90 per cent; Cl = 25.61 per cent.

Iodoacetylaminoethanol. 1 mol. of chloroacetylaminoethanol was dissolved in a solution of 1.1 mols. of sodium iodide in dry acetone and allowed to stand for twenty-four hours. The precipitated sodium chloride was filtered off and the filtrate con-

centrated to a syrup. Although all attempts at further purification were unsuccessful, the crude product was sufficiently pure for the preparation of the two following salts.

Iodoacetylaminoethanol and hexamethylenetetramine. The crude iodo compound was dissolved in absolute alcohol and added to a cold solution of the base in the minimum amount of absolute alcohol. The salt, which soon began to separate, was filtered off after twenty-four hours and washed with absolute alcohol. It melts with decomposition at 142–3°.

0.1968 gm. of substance required 5.43 cc. $AgNO_3$ Solution I.
Calculated for $C_{10}H_{20}O_2N_5I$: I = 34.35 per cent.
Found: I = 34.73 per cent.

Iodoacetylaminoethanol and trimethylamine. For purposes of comparison this salt was prepared in the same way as the hexamethylenetetraminium compound. It sinters at 135° and melts at 138–9° (corrected). It dissolves readily in water and warm alcohol.

0.1954 gm. of substance required 6.85 cc. $AgNO_3$ Solution I.
Calculated for $C_7H_{17}O_2N_2I$: I = 44.05 per cent.
Found: I = 44.14 per cent.

Chloroacetylaminoethyl benzoate. In addition to the method given above for the preparation of esters of chloroacetylaminoethanol, the Schotten-Baumann method, starting with aminoethyl benzoate hydrobromide and chloroacetyl chloride, may be employed. The yield resulting from this procedure is poor, however, owing to the rapid rearrangement of the free base to the oxyethylbenzamide. Recrystallized first from absolute alcohol, then toluene, the ester forms flat needles which soften at 68.5° and melt at 69–9.5° (corrected). It is very soluble in chloroform, less so in benzene and ether.

0.2135 gm. of substance (Kjeldahl) required 8.8 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{11}H_{12}O_3NCl$: N = 5.80 per cent.
Found: N = 5.77 per cent.

Chloroacetylaminoethyl benzoate and hexamethylenetetramine. Equimolecular proportions of the components were heated in a small amount of dry chloroform for one hour. The addition

of dry acetone resulted in the precipitation of the salt as a paste. This became crystalline on warming and rubbing. The compound darkens at about 158° and melts with decomposition at 162°.

0.2006 gm. of substance required 5.5 cc. $AgNO_3$ Solution I.
Calculated for $C_{17}H_{24}O_3N_5Cl$: Cl = 9.29 per cent.
Found: Cl = 9.65 per cent.

Chloroacetylaminoethyl o-toluate. Recrystallized successively from 50 per cent alcohol, absolute alcohol, and toluene, the ester forms faintly pinkish clusters of spears which soften at 64.5° and melt at 65–5.5° (corrected), the melting point being unchanged by a subsequent recrystallization from absolute alcohol. It is extremely soluble in chloroform, less so in ether and benzene.

0.2065 gm. of substance (Kjeldahl) required 8.25 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{12}H_{14}O_3NCl$: N = 5.48 per cent.
Found: N = 5.60 per cent.

Chloroacetylaminoethyl o-toluate and hexamethylenetetramine. After one hour's boiling in dry chloroform solution the salt separated on cooling. It melts at 168° with decomposition.

0.2096 gm. of substance required 5.38 cc. $AgNO_3$ Solution I.
Calculated for $C_{18}H_{26}O_3N_5Cl$: Cl = 8.96 per cent.
Found: Cl = 9.03 per cent.

Chloroacetylaminoethyl p-toluate. Recrystallized first from absolute alcohol, then toluene, the ester forms glistening, felted hairs which are rather less soluble in the usual organic solvents than is the *ortho* isomer. It melts at 106–7.5° (corrected).

0.2081 gm. of substance (Kjeldahl) required 8.1 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{12}H_{14}O_3NCl$: N = 5.48 per cent.
Found: N = 5.45 per cent.

Chloroacetylaminoethyl p-toluate and hexamethylenetetramine. After one hour's boiling, the salt was precipitated from the chloroform solution by means of dry acetone. When rapidly heated it melts to an orange liquid at 161–2°, but melts at a much lower temperature when slowly heated.

0.2030 gm. of substance required 5.22 cc. $AgNO_3$ Solution I.
Calculated for $C_{18}H_{26}O_3N_5Cl$: Cl = 8.96 per cent.
Found: Cl = 9.05 per cent.

Chloroacetylaminoethyl β-naphthoate. β-Naphthoyl chloride was obtained in excellent yield from the acid and thionyl chloride. The ester prepared with its aid, when recrystallized first from absolute alcohol, then twice from benzene, forms minute, voluminous, strongly triboëlectric needles which melt at 137–9° (corrected). This method of purification removes an impurity crystallizing in the form of plates, which was not further investigated. The ester is sparingly soluble in cold acetone, readily in acetic acid.

0.1877 gm. of substance (Kjeldahl) required 6.7 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{15}H_{14}O_3NCl$: N = 4.80 per cent.
Found: N = 5.00 per cent.

Chloroacetylaminoethyl β-naphthoate and hexamethylenetetramine. After concentration of the chloroform solution to small bulk, precipitation of the salt was completed by the addition of several volumes of dry acetone. The compound was purified by boiling for one hour with dry acetone. It melts and decomposes at 174–6°. When once isolated it is difficultly soluble in chloroform.

0.2092 gm. of substance required 4.84 cc. $AgNO_3$ Solution I.
Calculated for $C_{21}H_{26}O_3N_5Cl$: Cl = 8.22 per cent.
Found: Cl = 8.14 per cent.

Chloroacetylaminoethyl o-nitrobenzoate. *o*-Nitrobenzoyl chloride prepared from the acid and thionyl chloride was used. As the crude ester could not be made to crystallize, it was taken up with ether, washed with small amounts of 10 per cent sulphuric acid until all pyridine had been removed, then with 5 per cent sodium carbonate solution, and finally with water. After drying over sodium sulphate, the solvent was removed, finally *in vacuo*, but the resulting syrup could not be obtained crystalline. It is miscible in all proportions with chloroform and is insoluble in ligroin.

0.2034 gm. of substance (Kjeldahl) required 13.60 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{11}H_{11}O_5N_2Cl$: N = 9.78 per cent.
Found: N = 9.37 per cent.

Chloroacetylaminoethyl o-nitrobenzoate and hexamethylenetetramine. The solution of the components in dry, boiling chloroform

soon set to a solid cake. This was disintegrated, after which the heating was continued for one-half hour. Additional quantities of the salt were obtained from the mother liquors on continued boiling. As precipitated, it contains chloroform of crystallization, one molecule of which is retained even on heating *in vacuo* at the temperature of boiling alcohol over sulphuric acid. A strong odor of chloroform is apparent when the salt is dissolved in water.

0.3590 gm. of substance required 6.89 cc. $AgNO_3$ Solution I.
Calculated for $C_{17}H_{23}O_5N_6Cl.CHCl_3$: $Cl^- = 6.49$ per cent.
Found: $Cl^- = 6.75$ per cent.

The chloroform of crystallization may be removed by boiling the salt for one hour in dry acetone. It then melts with decomposition at 174° when plunged into the bath at 165°.

0.2094 gm. of substance required 4.98 cc. $AgNO_3$ Solution I.
Calculated for $C_{17}H_{23}O_5N_6Cl$: Cl = 8.31 per cent.
Found: Cl = 8.37 per cent.

Chloroacetylaminoethyl m-nitrobenzoate. The ester could not be obtained crystalline. Even when purified as in the case of the *ortho* isomer it was still very impure, as shown by analysis.

Chloroacetylaminoethyl m-nitrobenzoate and hexamethylenetetramine. The chloroform solution was boiled for about two and a half hours, filtered from a small amount of what was probably hexamethylenetetraminium chloride, and treated with dry acetone. The salt separated as an oil which soon crystallized. The product was filtered off and warmed several hours with dry acetone.

0.2273 gm. of substance required 5.96 cc. $AgNO_3$ Solution I.
Calculated for $C_{17}H_{23}O_5N_6Cl$: Cl = 8.31 per cent.
Found: Cl = 9.23 per cent.

Chloroacetylaminoethyl p-nitrobenzoate. Recrystallized from absolute alcohol, the ester forms long, prismatic needles which melt at 127–8° (corrected). It is difficultly soluble in cold absolute alcohol and benzene, more easily in acetone and chloroform.

0.1969 gm. of substance required 6.8 cc. $AgNO_3$ Solution I.
Calculated for $C_{11}H_{11}O_5N_2Cl$: Cl = 12.38 per cent.
Found: Cl = 12.16 per cent.

Chloroacetylaminoethyl p-nitrobenzoate and hexamethylenetetramine. The salt separates from the chloroform solution. It melts at 182–2.5° with decomposition.

0.1985 gm. of substance required 4.55 cc. $AgNO_3$ Solution I.
Calculated for $C_{17}H_{23}O_5N_6Cl$: Cl = 8.31 per cent.
Found: Cl = 8.07 per cent.

Chloroacetylaminoethyl p-nitrobenzoate and trimethylamine. The ester and an excess of trimethylamine were heated in dry chloroform at 100° for one hour in a sealed tube. The resulting clear solution was evaporated to small bulk and treated first with a little dry acetone, then with dry ether. The salt separated as a white, crystalline powder melting at 175–6° (corrected). When once isolated it is insoluble in chloroform and acetone, but dissolves easily in water and in alcohol.

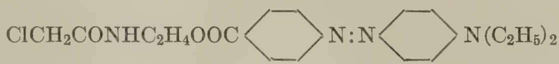
0.1473 gm. of substance required 4.3 cc. $AgNO_3$ Solution I.
Calculated for $C_{14}H_{20}O_5N_3Cl$: Cl = 10.26 per cent.
Found: Cl = 10.28 per cent.

Chloroacetylaminoethyl p-aminobenzoate. 12 grams of chloroacetylaminoethyl *p*-nitrobenzoate were added slowly to a solution of 50 grams of stannous chloride in 100 cc. of acetic acid saturated with hydrochloric acid gas. The temperature rose rapidly, but was kept below 30° with the aid of a freezing mixture. The reaction mixture, from which a double salt had partially separated as a gummy mass, was poured into ice water and the solution treated with 50 per cent sodium hydroxide solution until the tin precipitate redissolved, taking care that the solution remained ice cold in order to prevent saponification of the ester. In a second experiment the ester crystallized out at this point in the form of glistening platelets which were immediately filtered off, but in the first trial it was necessary to shake out the alkaline solution with chloroform in order to isolate the compound. After drying the chloroform solution over sodium sulphate it was concentrated to a syrup. When this was taken up in a little hot alcohol and cooled, it set to a solid cake. This was broken up with a little dry ether and filtered. Yield: 7 grams. The ester was recrystallized twice from absolute alcohol, using bone-black the first time. It forms

pale cream-colored, minute spindles which melt at 117–8.5° (corrected) with preliminary softening. It is very difficultly soluble in ether, benzene, toluene, and cold acetone.

0.1514 gm. of substance (Kjeldahl) required 11.55 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{11}H_{13}O_3N_2Cl$: N = 10.92 per cent.
Found: N = 10.69 per cent.

Chloroacetylaminoethyl p-[azodiethylaniline]-benzoate (chloroacetylaminoethyl ester of p-carboxybenzeneazo-p′-diethylaminobenzene).



4 grams of chloroacetylaminoethyl *p*-aminobenzoate were dissolved at 0° in 35 cc. of normal hydrochloric acid. With continued cooling the solution was diazotized with a solution of 1.1 grams of sodium nitrite in a little water. After stirring for ten minutes, sodium acetate was added until the reaction was neutral to Congo Red paper, after which a solution of 4.8 grams of diethylaniline in 50 cc. of alcohol was added. Coupling commenced at once, with separation of a tar which partially crystallized on scratching, forming microscopic rosettes of platelets. The addition of one-half volume of water completed the separation of the dye and resulted in the crystallization of the entire product. This was filtered off, washed with 50 per cent alcohol, and dissolved in a little hot absolute alcohol. The substance separated as an oil on cooling, but was converted into aggregates of red, microscopic crystals by the cautious addition of water, accompanied by rubbing. After standing in the ice box over night the dye was filtered off, washed with 50 per cent alcohol, and dried. The yield was practically quantitative. The dye sinters at 111° and gradually melts until it is completely fluid at 116°.

0.1691 gm. of substance (Kjeldahl) required 16.36 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{21}H_{25}O_3N_4Cl$: N = 13.45 per cent.
Found: N = 13.56 per cent.

Chloroacetylaminoethyl p-[azodiethylaniline]-benzoate and hexamethylenetetramine. Equimolecular amounts of the components were boiled in a little dry chloroform for one-half hour, after which the mixture set to a thick paste. By the cautious addition of

dry acetone this was converted into a light orange, crystalline mass. As first obtained, this seemed to be entirely soluble in water, but when dried the salt was only incompletely soluble. It melts at 179–81°. The aqueous solution dyes silk a bright orange shade.

0.3163 gm. of substance gave 0.0828 gm. AgCl.
Calculated for $C_{27}H_{37}O_3N_8Cl$: Cl = 6.37 per cent.
Found: Cl = 6.47 per cent.

Chloroacetylaminoethyl acetylsalicylate. The acetylsalicylic chloride used for this preparation was prepared according to Anschütz.[7] The crude ester was dried *in vacuo* and recrystallized from benzene, forming glistening, snow-white tablets, melting at 117° (corrected) with slight preliminary softening. It is soluble in the cold in absolute alcohol, difficultly in ethyl acetate.

0.2033 gm. of substance (Kjeldahl) required 7.0 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{13}H_{14}O_5NCl$: N = 4.73 per cent.
Found: N = 4.82 per cent.

Chloroacetylaminoethyl acetylsalicylate and hexamethylenetetramine. The clear chloroform solution obtained after boiling for one and a half hours was evaporated to small bulk. Several volumes of dry acetone were then added. The salt remained in solution until this was seeded with crystals obtained in a test portion by the addition of dry ether. The product forms microplatelets which melt to a yellowish liquid at 154–6° with slight gas evolution. It dissolves readily in chloroform, alcohol, and water.

0.2180 gm. of substance required 5.14 cc. $AgNO_3$ Solution I.
Calculated for $C_{19}H_{26}O_5N_5Cl$: Cl = 8.07 per cent.
Found: Cl = 8.30 per cent.

Chloroacetylaminoethyl anisate (chloroacetylaminoethyl p-methoxybenzoate). In addition to the usual method of preparation, this ester may be obtained, although in poor yield, from aminoethyl anisate hydrobromide and chloroacetyl chloride by the Schotten-Baumann method. Recrystallized first from dilute alcohol, then benzene containing a little toluene to prevent its freezing in the ice box, and finally from absolute alcohol, the ester melts

[7] R. Anschütz: *Ann. d. Chem.*, ccclxvii, p. 173, 1909.

constantly at 96.5–7° (corrected) with slight preliminary softening. It forms rosettes of needles which are rather difficultly soluble in cold ether, benzene, and alcohol, but dissolve readily in chloroform.

0.1830 gm. of substance (Kjeldahl) required 6.85 cc. $\frac{N}{10}$ HCl.
0.1951 gm. of substance required 13.58 cc. $AgNO_3$ Solution II.
Calculated for $C_{12}H_{14}O_4NCl$: N = 5.16 per cent; Cl = 13.05 per cent.
Found: N = 5.24 per cent; Cl = 12.95 per cent.

Chloroacetylaminoethyl anisate and hexamethylenetetramine. After one hour's boiling the salt was precipitated from the chloroform solution by means of dry acetone. It melts at 153–4° with decomposition and gives a pale pink color in sulphuric acid.

0.1528 gm. of substance required 3.7 cc. $AgNO_3$ Solution I.
Calculated for $C_{18}H_{26}O_4N_5Cl$: Cl = 8.61 per cent.
Found: Cl = 8.52 per cent.

Chloroacetylaminoethyl cinnamate. Recrystallized first from absolute alcohol, then toluene, then absolute alcohol, the ester melts constantly at 120–2° (corrected). It forms delicate, glistening needles which are difficultly soluble in the cold in ether, alcohol, benzene, and toluene.

0.2677 gm. of substance (Kjeldahl) required 10.08 cc. $\frac{N}{10}$ HCl.
0.1456 gm. of substance required 10.05 cc. $AgNO_3$ Solution II.
Calculated for $C_{13}H_{14}O_3NCl$: N = 5.24 per cent; Cl = 13.25 per cent.
Found: N = 5.26 per cent; Cl = 12.88 per cent.

Chloroacetylaminoethyl ethyl ether, $ClCH_2CONHCH_2CH_2OC_2H_5$. 25 cc. of two-normal sodium hydroxide solution were added to a cooled solution of 3 grams of freshly distilled aminoethyl ethyl ether[8] in a little water. The mixture was chilled with salt and ice and chloroacetylated with 2 mols. of chloroacetyl chloride, adding alkali from time to time as necessary. At the end, solid potassium carbonate was added until the chloroacetyl derivative separated as an oil. This was taken up in chloroform and the aqueous layer extracted five times with the same solvent. The chloroform solution was dried over sodium sulphate and evaporated to small bulk. Almost all of the residue boiled at 132° (corrected) at 15 mm. Yield: 3.5 grams.

[8] L. Knorr: *Ber. d. deutsch. chem. Gesellsch.*, xxxvii, p. 3506, 1904.

0.1266 gm. of substance (Kjeldahl) required 7.55 cc. $\frac{N}{10}$ HCl.
0.0957 gm. of substance required 11.13 cc. $AgNO_3$ Solution II.
Calculated for $C_6H_{12}O_2NCl$: N = 8.46 per cent; Cl = 21.41 per cent.
Found: N = 8.35 per cent; Cl = 21.64 per cent.

Chloroacetylaminoethyl ethyl ether and hexamethylenetetramine. The salt was precipitated from the chloroform solution by the addition of dry acetone and rubbing. It was filtered off, washed with dry acetone, and rapidly transferred to a desiccator as it proved to be very hygroscopic. It melted unsharply at 135–45°.

0.1979 gm. of substance required 12.2 cc. $AgNO_3$ Solution II.
Calculated for $C_{12}H_{24}O_2N_5Cl$: Cl = 11.60 per cent.
Found: Cl = 11.47 per cent.

Aminoethyl o-tolyl ether (o-methylphenoxyethylamine),

o-$CH_3C_6H_4OC_2H_4NH_2$.

60 grams of *o*-methylphenoxyethyl bromide were heated at 120° for four hours in an autoclave with 600 cc. of alcoholic ammonia saturated at 0°. The ammonia and alcohol were boiled off, finally with steam, leaving a solution containing oil droplets. These (probably the imino compound) were removed by shaking out with ether. The aqueous layer was then made alkaline and shaken out again with ether. The residue obtained after evaporating off the ether was fractionated *in vacuo*. The amine boils at 128° (corrected) at 19 mm. and has an unpleasant odor. It is almost insoluble in cold water, but dissolves more freely on heating. It gives a pale pinkish color in sulphuric acid.

0.2542 gm. of substance (Kjeldahl) required 16.85 cc. $\frac{N}{10}$ HCl.
Calculated for $C_9H_{13}ON$: N = 9.27 per cent.
Found: N = 9.28 per cent.

Chloroacetylaminoethyl o-tolyl ether. To a solution of 10 grams of the amine in benzene were added an excess of two-normal, aqueous sodium hydroxide and, with cooling and vigorous shaking, a solution of 10 grams of chloroacetyl chloride in dry benzene. The mixture was then acidified and the benzene layer washed with water and sodium carbonate solution. After drying it was evaporated to small bulk. The resulting syrup (14 grams), which could not be made to crystallize, was fractionated *in vacuo*. The substance boiled at 168–9° at 0.7 mm. It crystallized on standing, and,

after recrystallization from a little toluene with the aid of ligroin, formed minute prisms softening at 39° and melting at 39.5–40.5° (corrected). It gives a faint, pinkish color with sulphuric acid.

0.2589 gm. of substance (Kjeldahl) required 11.40 cc. $\frac{N}{10}$ HCl.
0.2133 gm. of substance required 17.4 cc. $AgNO_3$ Solution II.
Calculated for $C_{11}H_{14}O_2NCl$: N = 6.15 per cent; Cl = 15.57 per cent.
Found: N = 6.17 per cent; Cl = 15.18 per cent.

Chloroacetylaminoethyl o-tolyl ether and hexamethylenetetramine. Equimolecular amounts of the components were boiled for one hour in dry chloroform. As the salt did not separate, the solution was evaporated to small bulk and treated with dry acetone. This caused the salt to separate, after which the mixture was warmed until the product became entirely crystalline. It melts at 162° to a dark liquid and gives a bright red color with sulphuric acid.

0.1946 gm. of substance required 9.55 cc. $AgNO_3$ Solution II.
Calculated for $C_{17}H_{26}O_2N_5Cl$: Cl = 9.64 per cent.
Found: Cl = 9.12 per cent.

Chloroacetylethylaminoethanol, $ClCH_2CON(C_2H_5)C_2H_4OH$. 6 grams of ethylaminoethanol were chloroacetylated in the same manner as was aminoethanol. The reaction mixture was saturated with sodium carbonate and shaken out ten times with chloroform. This was dried and evaporated to small bulk, finally *in vacuo*. Yield of crude product: 5 grams. Roughly three-quarters of this boiled at 130–5° at 0.6 mm., yielding a pale yellow, viscous oil which was soluble in and miscible with water.

0.2729 gm. of substance (Kjeldahl) required 16.45 cc. $\frac{N}{10}$ HCl.
0.1967 gm. of substance required 21.3 cc. $AgNO_3$ Solution II.
Calculated for $C_6H_{12}O_2NCl$: N = 8.46 per cent; Cl = 21.41 per cent.
Found: N = 8.44 per cent; Cl = 20.14 per cent.

Chloroacetylethylaminoethyl p-nitrobenzoate. As the crude ester did not crystallize it was shaken out from the acid mixture with chloroform. This was washed, first with water, then with dilute sodium carbonate solution, then water, dried, and concentrated to a syrup. This was taken up in a little hot alcohol and allowed to stand, when it gradually crystallized. Recrystallized, with bone-blacking, first from absolute alcohol, then toluene, and finally absolute alcohol, it forms almost colorless, minute rhombs

which melt at 73.5–4.° (corrected) with slight preliminary softening. It is difficultly soluble in the cold in ether, absolute alcohol, and toluene, but dissolves readily in benzene and chloroform.

0.1946 gm. of substance required 11.72 cc. $AgNO_3$ Solution II.
Calculated for $C_{13}H_{15}O_5N_2Cl$: Cl = 11.27 per cent.
Found: Cl = 11.20 per cent.

Chloroacetylethylaminoethyl p-nitrobenzoate and hexamethylenetetramine. The chloroform solution, after boiling for one and a half hours, was filtered from a little hexamethylenetetraminium chloride and concentrated to small bulk. The salt separated after the addition of several volumes of dry acetone and scratching. After standing over night it was filtered off and washed with dry acetone. It melts at 154–5° to an orange liquid and is difficultly soluble in water.

0.1394 gm. of substance required 2.9 cc. $AgNO_3$ Solution I.
Calculated for $C_{19}H_{27}O_5N_6Cl$: Cl = 7.80 per cent.
Found: Cl = 7.33 per cent.

Chloroacetylphenylaminoethanol, $ClCH_2CON(C_6H_5)C_2H_4OH$. 20 grams of phenylaminoethanol (oxyethylaniline) were dissolved in benzene and chloroacetylated by means of chloroacetyl chloride in the presence of two-normal sodium hydroxide. The benzene layer was washed successively with water, dilute hydrochloric acid (which removed considerable unchanged amine), and water. After drying, the benzene was evaporated to small bulk and treated with ligroin, precipitating an oil which gradually crystallized. This was recrystallized twice from warm benzene by adding ligroin until a permanent turbidity appeared. The compound forms prisms which melt at 67.5–8.5° (corrected) with slight preliminary softening.

0.1998 gm. of substance (Kjeldahl) required 9.14 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{10}H_{12}O_2NCl$: N = 6.56 per cent.
Found: N = 6.41 per cent.

Chloroacetylphenylaminoethyl p-nitrobenzoate. The ester was recrystallized three times from absolute alcohol, forming faintly yellow spears which melt at 115–6° (corrected) with slight preliminary softening. It is rather soluble in benzene, very difficultly in ether.

0.2099 gm. of substance gave 14.85 cc. N (767 mm. and 30.3°).
Calculated for $C_{17}H_{15}O_5N_2Cl$: N = 7.73 per cent.
Found: N = 7.70 per cent.

Chloroacetylphenylaminoethyl p-nitrobenzoate and hexamethylenetetramine. After three hours' boiling, dry acetone was added to the chloroform solution. The salt crystallized slowly on scratching. After several hours in the ice box it was filtered off and washed with dry acetone. Yield: about 40 per cent of the theory. The compound melts at 167–8° to an orange paste.

0.1998 gm. of substance required 7.45 cc. $AgNO_3$ Solution II.
Calculated for $C_{23}H_{27}O_5N_6Cl$: Cl = 7.05 per cent.
Found: Cl = 6.93 per cent.

Owing to the failure of attempts to obtain crystalline hexamethylenetetraminium salts of chloroacetylphenylaminoethanol and the corresponding iodo-compound the bromoacetyl derivative and several of its esters were prepared. Great difficulty was experienced in obtaining these substances and their hexamethylenetetraminium salts in crystalline form. This applies also to several homologs which we attempted to prepare. The following substance is the only one of this series obtained in a state of purity.

Bromoacetylphenylaminoethanol. 30 grams of phenylaminoethanol were dissolved in 150 cc. of benzene, chilled, and slowly treated with a solution of 22 grams (0.5 mol.) of bromoacetyl bromide in dry benzene. The hydrobromide of the base separated as an oil. The mixture was shaken out successively with dilute hydrochloric acid, water, dilute sodium carbonate solution, and water. After drying, the benzene was evaporated to small bulk and carefully treated with ligroin. The crude bromoacetyl derivative separated on standing and scratching. Yield: 15 grams. When recrystallized from dry ether, rhombic crystals separated first, followed by warty masses. These were mechanically separated from the rhombs and recrystallized from absolute alcohol. This substance forms delicate needles melting at 131–3° with preliminary softening. It contains bromine, but was not further investigated. The rhombs were recrystallized from dry ether and then melted at 58–60° (corrected) with slight preliminary softening. These proved to be the desired bromoacetylphenylaminoethanol. It is very soluble in benzene and alcohol and slowly decomposes when exposed to the air.

0.2110 gm. of substance required 15.56 cc. $AgNO_3$ Solution II.
Calculated for $C_{10}N_{12}O_2NBr$: Br = 30.97 per cent.
Found: Br = 30.91 per cent.

p-Dimethylaminophenylaminoethanol,

p-$(CH_3)_2NC_6H_4NHC_2H_4OH$.

56 grams of *p*-aminodimethylaniline, 32 grams of ethylene chlorohydrin, and 80 cc. of water were boiled for two hours under a reflux. The resulting mixture was made alkaline with sodium hydroxide and shaken out with ether. This was dried, evaporated to small bulk, and fractionated *in vacuo*. 22 grams of the amino-alcohol distilled at 197–204° at 9 mm. and crystallized on cooling. Of this 14 grams boiled at 215–7° (corrected) at 16 mm. Recrystallized from toluene, it forms almost colorless aggregates of irregular plates melting at 32.5–5.° (corrected). It is difficultly soluble in toluene at 0° and in ligroin, but dissolves easily in the other organic solvents. It is hygroscopic and dissolves in water. The aqueous solution becomes violet on exposure to the air and gives, with a drop of potassium dichromate solution, a purple color followed by a violet precipitate.

0.2361 gm. of substance (Kjeldahl) required 25.45 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{10}H_{16}ON_2$: N = 15.55 per cent.
Found: N = 15.10 per cent.

Chloroacetyl-p-dimethylaminophenylaminoethanol.

The base was chloroacetylated in benzene solution by means of chloroacetyl chloride in the presence of two-normal sodium hydroxide solution. The benzene layer was washed with water, dried over sodium sulphate, and evaporated to small bulk. The chloroacetyl derivative crystallized on adding ligroin and scratching. Yield: 40–50 per cent of the theory. Recrystallized twice from absolute alcohol, it forms practically colorless rhombs which melt constantly at 85.5–7.° (corrected) with preliminary softening. It is difficultly soluble in cold toluene, alcohol, and ether.

0.0588 gm. of substance gave 5.6 cc. N (768 mm. and 20°).
0.1999 gm. of substance (Carius) gave 0.1109 gm. AgCl.
Calculated for $C_{12}H_{18}O_2N_2Cl$: N = 10.97 per cent; Cl = 13.87 per cent.
Found: N = 10.96 per cent; Cl = 13.72 per cent.

p-Methoxyphenylaminoethanol. 50 grams of *p*-anisidine, 16 grams of ethylene chlorohydrin, and 40 cc. of water were boiled for two hours under a reflux. The resulting mixture was made alkaline with sodium hydroxide and extracted three times with ether. This was rapidly dried over sodium sulphate and evaporated to small bulk. The residue, fractionated *in vacuo*, yielded 17 grams of the aminoalcohol boiling at 187–97° at 8–8.5 mm., of which most boiled at 188–91° at 8 mm. The distillate crystallized in the receiver. A portion recrystallized from dry, alcohol-free ether with the aid of a freezing mixture melted at 43.5–4.5° (corrected).

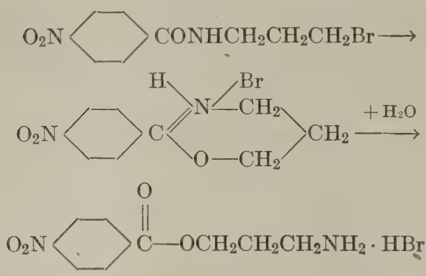
0.0953 gm. of substance (Kjeldahl) required 5.8 cc. $\frac{N}{10}$ HCl.
Calculated for $C_9H_{13}O_2N$: N = 8.38 per cent.
Found: N = 8.53 per cent.

(3) *Derivatives of γ-aminopropanol.*

γ-Bromopropyl-p-nitrobenzamide, $BrC_3H_6NHCOC_6H_4NO_2$. A solution of 18 grams of *p*-nitrobenzoyl chloride in benzene was added to a solution of 20 grams of γ-bromopropylamine hydrobromide in 100 cc. of water. With chilling and shaking, 100 cc. of two-normal sodium hydroxide solution were then added in small portions. The amide separated immediately, and was completely precipitated by adding ligroin. Yield: 25 grams. Recrystallized twice from benzene, then once from dry, alcohol-free chloroform, it forms minute, colorless needles which melt at 107.5–8.° (corrected) with slight preliminary softening. It is soluble in alcohol at room temperature.

0.1741 gm. of substance gave 0.1137 gm. AgBr.
Calculated for $C_{10}H_{11}O_3N_2Br$: Br = 27.83 per cent.
Found: Br = 27.79 per cent.

γ-Aminopropyl p-nitrobenzoate hydrobromide. 20 grams of the above amide were dissolved by heating with water on the water bath. The solution was finally boiled for several hours, completing the rearrangement according to the following scheme:[3]



The resulting solution was concentrated as far as possible on the water bath, forming a solid, crystalline cake on cooling. The hydrobromide was recrystallized from 95 per cent alcohol. Yield: 16 grams. It melts at 185–5.5° (corrected) and is readily soluble in water.

0.2549 gm. of substance required 8.25 cc. $AgNO_3$ Solution I.
Calculated for $C_{10}H_{12}O_4N_2 \cdot HBr$: Br = 26.19 per cent.
Found: Br = 25.67 per cent.

γ-Oxypropyl-p-nitrobenzamide. On dissolving γ-aminopropyl *p*-nitrobenzoate hydrobromide in water and adding 1 mol. of sodium hydroxide the free base separates as an oil. This is very unstable, however, and gradually rearranges to the γ-oxypropyl-*p*-nitrobenzamide, losing its solubility in acids and changing to a mass of heavy, rhombic prisms. Recrystallized from toluene, the amide melts at 102.5–3.5° (corrected). It is very difficultly soluble in dry ether and benzene, more soluble in chloroform, and readily so in absolute alcohol.

0.1619 gm. of substance gave 18.1 cc. N (762 mm. and 23.8°).
Calculated for $C_{10}H_{12}O_4N_2$: N = 12.50 per cent.
Found: N = 12.52 per cent.

γ-p-Nitrobenzoylaminopropyl chloroacetate. Equimolecular amounts of γ-oxypropyl-*p*-nitrobenzamide and chloroacetic anhydride were dissolved in chloroform and boiled for one hour. The solution was washed first with dilute sodium carbonate solution, then with water, and finally dried. The syrup which remained after concentration crystallized when treated with ligroin and rubbed. Recrystallized first from benzene containing a few drops of toluene, then from absolute alcohol, it forms faintly yellow, crystalline aggregates which melt from 93 to 106°.

0.2512 gm. of substance (Kjeldahl) required 16.6 cc. $\frac{N}{10}$ HCl.
0.1459 gm. of substance required 9.06 cc. $AgNO_3$ Solution II.
Calculated for $C_{12}H_{13}O_5N_2Cl$: N = 9.32 per cent; Cl = 11.80 per cent.
Found: N = 9.26 per cent; Cl = 11.55 per cent.

γ-Chloroacetylaminopropyl p-nitrobenzoate. Owing to the rapid rearrangement of γ-aminopropyl *p*-nitrobenzoate, it was found advisable, in order to obtain certain results, to chloroacetylate the base as follows: 5 grams of γ-aminopropyl *p*-nitrobenzoate hydrobromide in 25 cc. of water were chilled in a freezing mixture. To the suspension thus obtained was added a solution of 5 grams of chloroacetyl chloride in 50 cc. of dry, alcohol-free chloroform. The mixture was rapidly turbined, and, with continued cooling, treated drop by drop with 10 per cent sodium hydroxide solution until alkaline. The chloroform layer was then shaken out successively with dilute sodium hydroxide solution, dilute hydrochloric acid, and water. After drying it was concentrated to a syrup which crystallized when treated with ligroin. Recrystallized first from toluene, then twice from absolute alcohol, the ester forms aggregates of flat needles which melt constantly at 78–9° (corrected). It is difficultly soluble in ether and hot water, easily at room temperature in alcohol, benzene, and chloroform.

0.1649 gm. of substance gave 14.20 cc. N (765 mm. and 24.5°).
0.1638 gm. of substance required 10.12 cc. $AgNO_3$ Solution II.
Calculated for $C_{12}H_{13}O_5N_2Cl$: N = 9.32 per cent; Cl = 11.80 per cent.
Found: N = 9.65 per cent; Cl = 11.48 per cent.

γ-Chloroacetylaminopropyl p-nitrobenzoate and hexamethylenetetramine. The salt separates from the chloroform solution. When rapidly heated it melts and decomposes at 178–9° with preliminary darkening.

0.2000 gm. of substance required 4.55 cc. $AgNO_3$ Solution I.
Calculated for $C_{18}H_{25}O_5N_6Cl$: Cl = 8.05 per cent.
Found: Cl = 8.01 per cent.

γ-Chloroacetylaminopropyl anisate (γ-chloroacetylaminopropyl p-methoxybenzoate). 11 grams of γ-aminopropyl anisate hydrobromide were dissolved in 100 cc. of water, chilled in a freezing mixture, and treated alternately with small portions of 20 per cent sodium hydroxide solution and chloroacetyl chloride (2 mols.).

The mixture was shaken out with chloroform. This was washed as usual, dried, and concentrated. The resulting syrup set to a solid, crystalline cake on adding ligroin and scratching. Yield: 6.5 grams. Recrystallized first from absolute alcohol, then toluene, the ester forms faintly pink, radiating masses of delicate hairs which soften at 72° and melt at 72.5–3° (corrected). It is rather difficultly soluble in the cold in dry ether and benzene.

0.1309 gm. of substance (Kjeldahl) required 4.65 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{13}H_{18}O_4NCl$: N = 4.90 per cent.
Found: N = 4.98 per cent.

γ-Chloroacetylaminopropyl anisate and hexamethylenetetramine. After one hour's boiling, dry acetone was added to the chloroform solution, precipitating the salt as glistening leaflets. It melts at 167–8° to an orange liquid.

0.1993 gm. of substance required 8.65 cc. $AgNO_3$ Solution II.
Calculated for $C_{19}H_{30}O_4N_5Cl$: Cl = 8.33 per cent.
Found: Cl = 8.07 per cent.

(4) *Derivatives of aminoisopropanol,* $H_2NCH_2CH(CH_3)OH$.

Chloroacetylaminoisopropanol, $ClCH_2CONHCH_2CH(CH_3)OH$. 12 grams of aminoisopropyl alcohol[9] were chloroacetylated in the same way as the previous aliphatic aminoalcohols. Solid potassium carbonate was added to the reaction mixture until the chloroacetyl derivative separated as an oil. This was shaken out with chloroform and the aqueous solution extracted nine times more. The residual oil weighed 20 grams. This was fractionated *in vacuo*. The main portion boiled at 131–2° (corrected) at 0.6 mm. and crystallized on standing. It melts at 33–4.5° (corrected).

0.2462 gm. of substance (Kjeldahl) required 16.25 cc. $\frac{N}{10}$ HCl.
0.1560 gm. of substance required 19.45 cc. $AgNO_3$ Solution II.
Calculated for $C_5H_{10}O_2NCl$: N = 9.24 per cent; Cl = 23.39 per cent.
Found: N = 9.24 per cent; Cl = 23.18 per cent.

[9] Prepared by hydrolyzing β-oxypropylbenzamide (P. Hirsch: *Ber. d. deutsch. chem. Gesellsch.*, xxiii, p. 970, 1890; Gabriel and Heymann: *loc. cit.*) in a sealed tube with 1:1 hydrochloric acid at 150° for four hours. The benzoic acid was filtered off and the filtrate evaporated to dryness, taken up in water, and saturated with potassium carbonate. The amine was drawn off, dried over potassium carbonate, and fractionated.

Chloroacetylaminoisopropanol and hexamethylenetetramine. The salt separates from the boiling chloroform solution. It decomposes at 171–2°.

0.2036 gm. of substance required 13.3 cc. $AgNO_3$ Solution II.
Calculated for $C_{11}H_{22}O_2N_5Cl$: Cl = 12.16 per cent.
Found: Cl = 12.15 per cent.

Chloroacetylaminoisopropyl p-nitrobenzoate. Recrystallized from absolute alcohol, in which it is very sparingly soluble in the cold, the ester forms very faintly yellow, silky needles which melt constantly at 133.5–4.5° (corrected) with slight preliminary softening. It is soluble in cold acetone, but sparingly so in cold toluene.

0.1418 gm. of substance gave 11.9 cc. N (756 mm. and 23°).
Calculated for $C_{12}H_{13}O_5N_2Cl$: N = 9.32 per cent.
Found: N = 9.36 per cent.

Chloroacetylaminoisopropyl p-nitrobenzoate and hexamethylenetetramine. After boiling the dry chloroform solution of the components for one hour, most of the chloroform was evaporated off. The addition of dry acetone to the residual solution resulted in the precipitation of a mixture of the salt and unchanged hexamethylenetetramine. This was boiled with a small amount of dry chloroform in order to remove the base and was then washed with dry chloroform and dry acetone. Yield: about 35 per cent of the theory. The salt forms very faintly yellow plates which darken above 170° and melt with decomposition at 180–2°. It is somewhat sparingly soluble in water.

0.1743 gm. of substance required 7.30 cc. $AgNO_3$ Solution II.
Calculated for $C_{18}H_{25}O_5N_6Cl$: Cl = 8.05 per cent.
Found: Cl = 7.79 per cent.

Aminoisopropyl p-nitrobenzoate hydrobromide. *p*-Nitrobenzoylaminoisopropyl bromide[10] was covered with hot water and boiled until completely dissolved. The solution was then concentrated as far as possible on the water bath. On cooling, the residue set to a solid cake which was recrystallized from alcohol. It melts at 221–2° and is rather difficultly soluble in cold water.

[10] A. Uedinck: *Ber. d. deutsch. chem. Gesellsch.*, xxxii, p. 978, 1899.

The salt was analyzed for amino nitrogen by the Van Slyke method.

0.2004 gm. of substance gave 16.5 cc. N (761 mm. and 22°).
0.1993 gm. of substance required 6.5 cc. $AgNO_3$ Solution I.
Calculated for $C_{10}H_{12}O_4N_2 \cdot HBr$: Amino N = 4.59%; Br = 26.19%.
Found: Amino N = 4.65%; Br = 25.85%.

Oxyisopropyl-p-nitrobenzamide,

$$HOCH(CH_3)CH_2NHCOC_6H_4NO_2.$$

When alkali is added to the aqueous solution of the above hydrobromide a transitory cloudiness appears, followed by the deposition of glistening platelets melting at 168–9°. This is not the free aminoisopropyl *p*-nitrobenzoate, as claimed by Uedinck,[10] but is oxyisopropyl-*p*-nitrobenzamide. It thus appears that the rearrangement takes place with greater rapidity in this case than in that of the previously discussed normal propyl isomers. The correctness of this interpretation is shown by the insolubility of the substance in dilute acids, by the fact that it contains no amino nitrogen, as determined by Van Slyke's method, and by the fact that it forms a chloroacetate (see below), not a chloroacetylamino derivative, as is shown by the absence of *p*-nitrobenzoic acid in the saponification products after gentle treatment with alcoholic sodium hydroxide, and by direct comparison with chloroacetylaminoisopropyl *p*-nitrobenzoate (see above).

p-Nitrobenzoylaminoisopropyl chloroacetate,

$$O_2NC_6H_4CONHCH_2CH(CH_3)OOCCH_2Cl.$$

13 grams of the above oxyisopropyl-*p*-nitrobenzamide were suspended in a small volume of dry, alcohol-free chloroform and treated with 7 grams of chloroacetyl chloride. The mixture was boiled under a reflux until a clear solution had been obtained and the evolution of hydrochloric acid gas had ceased. More chloroform was added and the mixture shaken out with dilute sodium hydroxide solution. After drying and concentrating, the substance was obtained in crystalline form by the addition of ligroin. Yield: 13 grams. Recrystallized first from absolute alcohol, then twice from toluene, and finally from absolute alco-

hol, the ester forms almost colorless plates and prisms melting at 89–94° (corrected). The ester is soluble in cold ether and acetone.

0.1447 gm. of substance (Kjeldahl) required 9.80 cc. $\frac{N}{10}$ HCl.
0.1509 gm. of substance required 9.27 cc. $AgNO_3$ Solution II.
Calculated for $C_{12}H_{13}O_5N_2Cl$: N = 9.32 per cent; Cl = 11.80 per cent.
Found: N = 9.49 per cent; Cl = 11.42 per cent.

p-Nitrobenzoylaminoisopropyl chloroacetate and hexamethylenetetramine. The salt separates from the hot chloroform solution as an oil which redissolves on cooling. It was obtained crystalline by adding several volumes of dry acetone and scratching. It melts with decomposition at 175–8°.

0.2381 gm. of substance required 5.55 cc. $AgNO_3$ Solution I.
Calculated for $C_{18}H_{25}O_5N_6Cl$: Cl = 8.05 per cent.
Found: Cl = 8.21 per cent.

(5) Derivatives of the higher aliphatic aminoalcohols.

δ-Chloroacetylamino-n-butanol, $ClCH_2CONHC_4H_8OH$. This was prepared from δ-aminobutanol[11] in the same way as the isopropyl homolog. The reaction mixture, after addition of potassium carbonate, was shaken out six times with chloroform. This was dried, evaporated, and concentrated to a syrup. Yield, from 12 grams of aminoalcohol: 19 grams. When fractionated *in vacuo,* the main portion boiled at 165–7° (corrected) at 0.7 mm. and crystallized on cooling. It melts at 30° (corrected) and is soluble in water, alcohol, and chloroform, but very sparingly so in benzene.

0.3001 gm. of substance (Kjeldahl) required 18.2 cc. $\frac{N}{10}$ HCl.
0.2465 gm. of substance required 28.56 cc. $AgNO_3$ Solution II.
Calculated for $C_6H_{12}O_2NCl$: N = 8.46 per cent; Cl = 21.41 per cent.
Found: N = 8.49 per cent; Cl = 21.55 per cent.

δ-Chloroacetylamino-n-butanol and hexamethylenetetramine. The salt gradually separated from the chloroform solution as an oil. On adding an equal volume of dry acetone and allowing to stand protected from moisture it gradually crystallized. The salt is hygroscopic. It sinters at 122° and melts at 125°.

[11] L. Henry: *Ber. d. deutsch. chem. Gesellsch.*, xxxiii, p. 3170, 1900.

0.2273 gm. of substance required 13.6 cc. $AgNO_3$ Solution II.
Calculated for $C_{12}H_{24}O_2N_5Cl$: Cl = 11.61 per cent.
Found: Cl = 11.13 per cent.

δ-Chloroacetylaminobutyl p-nitrobenzoate. The ester was recrystallized first from absolute alcohol, then toluene, forming practically white rosettes of minute prisms melting at 79° (corrected). It is soluble in the cold in ether and benzene, and extremely so in chloroform.

0.1489 gm. of substance gave 11.8 cc. N (760 mm. and 22.5°).
Calculated for $C_{13}H_{15}O_5N_2Cl$: N = 8.91 per cent.
Found: N = 8.91 per cent.

δ-Chloroacetylaminobutyl p-nitrobenzoate and hexamethylenetetramine. After one and one-half hours' boiling, the salt crystallized from the chloroform solution on cooling and scratching. It forms thick, faintly yellow plates which darken above 165° and melt to a thick, brown liquid at 170–2°. It is sparingly soluble in water.

0.1735 gm. of substance required 7.01 cc. $AgNO_3$ Solution II.
Calculated for $C_{19}H_{27}O_5N_6Cl$: Cl = 7.80 per cent.
Found: Cl = 7.51 per cent.

β-Chloroacetylamino-γ-butanol,

$$CH_3CH(OH)CH(NHCOCH_2Cl)CH_3.$$

β-Amino-γ-butanol (made by the reduction of methyl isonitrosoethyl ketone[12]) was chloroacetylated and isolated in the same way as in the previous cases. The crude product crystallized partially, but was purified by distillation *in vacuo*. It boils at 119° (corrected) at 0.3 mm. and solidifies on cooling to a crystalline mass which melts at 38–9° (corrected). It has a faint isonitrile-like odor, and is less readily soluble in benzene than in water, alcohol, and chloroform.

0.2397 gm. of substance (Kjeldahl) required 14.35 cc. $\frac{N}{10}$ HCl.
0.1017 gm. of substance required 11.63 cc. $AgNO_3$ Solution II.
Calculated for $C_6H_{12}O_2NCl$: N = 8.46 per cent; Cl = 21.41 per cent.
Found: N = 8.39 per cent; Cl = 21.27 per cent.

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[12] E. Strauss: *Ber. d. deutsch. chem. Gesellsch.*, xxxiii, p. 2827, 1900.

β-Chloroacetylamino-γ-butanol and hexamethylenetetramine. The salt separates from the boiling chloroform solution. It melts at 167–9°.

0.2100 gm. of substance required 13.4 cc. $AgNO_3$ Solution II.
Calculated for $C_{12}H_{24}O_2N_5Cl$: Cl = 11.61 per cent.
Found: Cl = 11.87 per cent.

β-Chloroacetylamino-γ-butyl p-nitrobenzoate. Recrystallized first from absolute alcohol, then toluene, the ester forms pale cream-colored rosettes of delicate leaflets which melt at 117–8° (corrected). It dissolves readily in cold acetone and chloroform, more sparingly, however, in ether. Yield: about 40 per cent of the theory.

0.1485 gm. of substance gave 11.8 cc. N (755 mm. and 21.5°).
Calculated for $C_{13}H_{15}O_5N_2Cl$: N = 8.91 per cent.
Found: N = 8.92 per cent.

β-Chloroacetylamino-γ-butyl p-nitrobenzoate and hexamethylenetetramine. The salt separates from the boiling chloroform solution of the components. It forms colorless micro-crystals which melt with decomposition at 189–91° and dissolve readily in water. Yield: about 45 per cent of the theory.

0.1689 gm. of substance required 6.83 cc. $AgNO_3$ Solution II.
Calculated for $C_{19}H_{27}O_5N_6Cl$: Cl = 7.80 per cent.
Found: Cl = 7.52 per cent.

γ-Chloroacetylamino-β-pentanol,

$$CH_3CH_2CH(NHCOCH_2Cl)CH(OH)CH_3.$$

5 grams of γ-amino-β-pentanol (prepared by reduction of methyl isonitrosopropyl ketone according to Jänicke's method[13]) were chloroacetylated in the usual manner. The crude product crystallized on cooling. Yield: 8 grams. It boils at 126–8° at 0.3 mm. and crystallizes on cooling to a waxy solid which melts at 52–60°. No suitable means of recrystallization could be found. The compound is somewhat hygroscopic and is

[13] E. Jänecke: *Ber. d. deutsch. chem. Gesellsch.*, xxxii, p. 1095, 1899.

easily soluble in water, alcohol, acetone, and chloroform, difficultly in benzene and ligroin.

0.2463 gm. of substance (Kjeldahl) required 14.05 cc. $\frac{N}{10}$ HCl.
Calculated for $C_7H_{14}O_2NCl$: N = 7.80 per cent.
Found: N = 7.99 per cent.

γ-Chloroacetylamino-β-pentanol and hexamethylenetetramine. Addition of the components proceeded slowly in chloroform solution. Several volumes of dry acetone were finally added, completing the precipitation. The salt was boiled out with dry acetone and dried. It melts and decomposes at 167–8° with preliminary darkening and is somewhat hygroscopic.

0.1932 gm. of substance required 12.1 cc. $AgNO_3$ Solution II.
Calculated for $C_{13}H_{26}O_2N_5Cl$: Cl = 11.09 per cent.
Found: Cl = 11.65 per cent.

Chloroacetylaminomethylmethylethyl carbinol (α-chloroacetylamino-β-methyl-β-butanol), $C_2H_5\underset{\displaystyle CH_3}{\underset{|}{C}}(OH)CH_2NHCOCH_2Cl$. Aminomethylmethylethyl carbinol[14] was chloroacetylated in the usual manner. The crude product was fractionated *in vacuo*, the portion taken boiling at 134° (corrected) at 0.7 mm. It was not obtained crystalline. It is soluble in water, alcohol, ether, acetone, chloroform, and benzene.

0.2600 gm. of substance (Kjeldahl) required 14.55 cc. $\frac{N}{10}$ HCl.
0.1700 gm. of substance required 17.75 cc. $AgNO_3$ Solution II.
Calculated for $C_7H_{14}O_2NCl$: N = 7.80 per cent; Cl = 19.74 per cent.
Found: N = 7.84 per cent; Cl = 19.41 per cent.

Chloroacetylaminomethylmethylethyl carbinol and hexamethylenetetramine. The salt separated from the boiling chloroform solution. It melts with decomposition at 170° and is somewhat hygroscopic.

0.2240 gm. of substance required 13.8 cc. $AgNO_3$ Solution II.
Calculated for $C_{13}H_{26}O_2N_5Cl$: Cl = 11.09 per cent.
Found: Cl = 11.46 per cent.

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[14] D. R.-P., 189481; *Friedlaenders Fortschr. d. Teerfarbenfabrikation*, viii, p. 1038, 1905–07.

γ-Chloroacetylamino-β-methyl-β-butanol,

$$\begin{array}{l} CH_3CH(NHCOCH_2Cl)C(OH)CH_3. \\ \qquad\qquad\qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\qquad\quad\;\; CH_3 \end{array}$$

15 grams of γ-amino-β-methyl-β-butanol[15] were chloroacetylated in the usual manner. The reaction mixture was neutralized and shaken out ten times with chloroform. The crude residue from the chloroform extract weighed 19 grams. When fractionated *in vacuo*, the main portion boiled at 122° (corrected) at 0.4 mm. and did not crystallize on cooling. It is soluble in water, alcohol, acetone, chloroform, and ether. It is miscible with a small amount of benzene but separates again when a larger quantity is added.

0.2148 gm. of substance (Kjeldahl) required 12.05 cc. $\frac{N}{10}$ HCl.
0.2235 gm. of substance required 23.22 cc. $AgNO_3$ Solution II.
Calculated for $C_7H_{14}O_2NCl$: N = 7.80 per cent; Cl = 19.74 per cent.
Found: N = 7.88 per cent; Cl = 19.33 per cent.

γ-Chloroacetylamino-β-methyl-β-butanol and hexamethylenetetramine. The product suddenly separated from the boiling chloroform solution. It melts with decomposition at 174–5°.

0.2285 gm. of substance required 13.2 cc. $AgNO_3$ Solution II.
Calculated for $C_{13}H_{26}O_2N_5Cl$: Cl = 11.10 per cent.
Found: Cl = 10.75 per cent.

(6) C-Aryl derivatives of aminoalcohols.

α-Phenyl-α-oxy-β-chloroacetylaminoethane,

$$C_6H_5CH(OH)CH_2NHCOCH_2Cl.$$

14.7 grams of α-phenyl-α-oxyethylamine were obtained by reducing 35 grams of benzaldehyde cyanohydrin in 50 per cent alcohol solution with 1400 grams of 4 per cent sodium amalgam, keeping the mixture just acid with acetic acid.[16] The crude product was suspended in 85 cc. of two-normal sodium hydroxide solution and diluted with enough water to dissolve most of the amine. 11 cc. of chloroacetyl chloride were then added in small amounts, with

[15] K. Krassousky: *Compt. rend. Acad. d. sc.*, cxlvi, p. 237, 1908.
[16] Compare D. R.-P. 193634; *Friedlaenders Fortschr. d. Teerfarbenfabrikation*, viii, p. 1183, 1905–07.

cooling and vigorous shaking. Most of the chloroacetyl derivative separated as an oil, an additional quantity being salted out by means of potassium carbonate. The mixture was extracted five times with chloroform. This was washed successively with dilute hydrochloric acid, water, and sodium carbonate solution. The chloroform solution, after drying over potassium carbonate, was evaporated to small bulk. The residue crystallized while still hot and was recrystallized from benzene containing a little ligroin. Yield: 10.2 grams. Recrystallized again from chloroform, it forms thick plates which soften at 108° and melt at 109–9.5° (corrected). It is soluble in hot water, hot chloroform, very difficultly in the cold in dry ether and chloroform, but easily in acetone.

0.2193 gm. of substance (Kjeldahl) required 10.35 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{10}H_{12}O_2NCl$: N = 6.56 per cent.
Found: N = 6.61 per cent.

α-Phenyl-α-oxy-β-chloroacetylaminoethane and hexamethylenetetramine. The salt separates from the boiling chloroform solution. It melts at 179° with decomposition.

0.1652 gm. of substance required 4.8 cc. $AgNO_3$ Solution I.
Calculated for $C_{16}H_{24}O_2N_5Cl$: Cl = 10.02 per cent.
Found: Cl = 10.23 per cent.

α-p-Tolyl-α-oxyethylamine, $CH_3C_6H_4CH(OH)CH_2NH_2$. 35 grams of crude *p*-tolualdehyde cyanohydrin (from the aldehyde bisulphite and potassium cyanide) were dissolved in 550 cc. of 50 per cent alcohol, cooled in a freezing mixture, and, with vigorous turbining, reduced with 1230 grams of 4 per cent sodium amalgam. During the process, which lasted several hours, the mixture was kept slightly acid with acetic acid.[16] The solution was then evaporated to about one-half volume. The solid mass of salts obtained on cooling was broken up, diluted with a little dilute hydrochloric acid, and filtered. A small amount of a semi-solid by-product remained with the salts. The filtrate was shaken out with ether to remove other impurities and was then covered with ether and made strongly alkaline with sodium hydroxide. After extracting four times with ether the ethereal solution was rapidly dried over potassium hydroxide and evaporated to small bulk. On cooling, the residue crystallized. Bone-

blacked and recrystallized from benzene, the amine forms snow-white crystals which melt at 68–9° (corrected). Yield: 9.1 grams. It is difficultly soluble in ether and cold benzene, easily in alcohol and water.

0.2160 gm. of substance (Kjeldahl) required 14.05 cc. $\frac{N}{10}$ HCl.
Calculated for $C_9H_{13}ON$: N = 9.27 per cent.
Found: N = 9.11 per cent.

α-p-Tolyl-α-oxy-β-chloroacetylaminoethane. 8.2 grams of the amine were chloroacetylated as in the case of the phenyl compound. The oily chloroacetyl derivative crystallized in the reaction mixture when seeded with a few crystals obtained from a test portion in 50 per cent alcohol by cooling in a freezing mixture. Yield: 5.1 grams after recrystallization from 50 per cent alcohol as above. Recrystallized again from benzene containing a little ligroin, it forms thick, colorless plates or prisms which melt at 81–2° (corrected) with slight preliminary softening. It is readily soluble in ether and chloroform.

0.2087 gm. of substance (Kjeldahl) required 9.4 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{11}H_{14}O_2NCl$: N = 6.16 per cent.
Found: N = 6.31 per cent.

α-p-Methoxyphenyl-α-oxyethylamine. 24 grams of anisaldehyde cyanohydrin (prepared from the aldehyde according to D. R.-P. 85230[17]) were reduced as in the previous examples. The solution was evaporated to about one-half volume, made more strongly acid with hydrochloric acid, and extracted twice with ether to remove impurities. After filtering from a small amount of insoluble material the aqueous solution was covered with ether and made strongly alkaline. After three extractions with ether this was rapidly dried over potassium hydroxide and evaporated to small bulk. The residue crystallized on cooling and was exhausted *in vacuo*. Yield: 6 grams. For analysis, a small portion was recrystallized from a mixture of benzene and ligroin.

0.1893 gm. of substance (Kjeldahl) required 10.75 cc. $\frac{N}{10}$ HCl.
Calculated for $C_9H_{13}O_2N$: N = 8.38 per cent.
Found: N = 7.95 per cent.

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[17] D. R.-P. 85230; *Friedlaenders Fortschr. d. Teerfarbenfabrikation*, iv, p. 160, 1894-97.

α,β-Diphenylchloroacetylaminoethanol,

$$\begin{array}{l} C_6H_5CHOH \\ \quad\;\, | \\ C_6H_5CHNHCOCH_2Cl. \end{array}$$

To a boiling solution of 5.6 grams of *α,β*-diphenylaminoethanol in dry benzene was added, drop by drop, a solution of 4.5 grams of chloroacetic anhydride in dry benzene. The solution was boiled for one hour, being allowed to concentrate at the same time to a volume of about 50 cc. The chloroacetyl derivative separated on cooling. The separation was completed by adding about two volumes of ligroin. After filtering, the product was recrystallized from absolute alcohol, forming interlaced, hair-like needles. Yield: 3.8 grams. It melts at 194–4.5° (corrected) and is difficultly soluble in cold absolute alcohol, chloroform, benzene, and ethyl acetate, but easily in acetone. It gives an olive-yellow color with sulphuric acid.

0.2907 gm. of substance (Kjeldahl) required 10.15 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{16}H_{16}O_2NCl$: N = 4.84 per cent.
Found: N = 4.89 per cent.

α,β-Diphenylchloroacetylaminoethanol and hexamethylenetetramine. The components reacted very slowly in boiling chloroform solution. After two hours the product was filtered off, washed with dry chloroform and dry acetone, and boiled for one hour with dry chloroform to complete the removal of the unchanged chloroacetyl derivative. Yield: poor. The salt forms hexagonal platelets containing solvent of crystallization which is lost on drying at 100° *in vacuo.* The compound then melts at 174° with decomposition. It gives a brown-orange color with sulphuric acid.

0.1991 gm. of substance required 4.54 cc. $AgNO_3$ Solution I.
Calculated for $C_{22}H_{28}O_2N_5Cl$: Cl = 8.25 per cent.
Found: Cl = 8.02 per cent.

The salt was obtained in better yield by heating the components with dry chloroform in a pressure bottle at 100° for one and one-quarter hours, but then required several hours' boiling with successive portions of dry acetone to free it from a water-insoluble impurity. After this treatment the salt contained acetone of crystallization, as was shown by the iodoform test. This was given off at 100° *in vacuo.*

α,β-Isodiphenylchloroacetylaminoethanol,

$$\begin{array}{l} C_6H_5CHOH \\ \quad\;| \\ C_6H_5CHNHCOCH_2Cl. \end{array}$$

10 grams of α,β-isodiphenylaminoethanol[18] were chloroacetylated in the same way as its isomer. During the addition of the chloroacetic anhydride a voluminous precipitate separated. This went into solution on adding more benzene and continuing the addition of the anhydride to the boiling solution. The mixture was then boiled for three-quarters of an hour, being concentrated at the same time. The crystals which separated on standing over night were filtered off and recrystallized from benzene and then from absolute alcohol. Yield: 2.5 grams. The compound forms colorless needles which melt at 156–7° (corrected) and are difficultly soluble in ether, readily in chloroform. It gives a greenish yellow color with sulphuric acid.

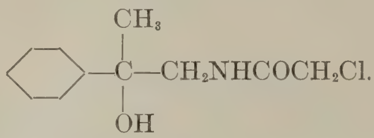
0.2463 gm. of substance (Kjeldahl) required 8.6 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{16}H_{16}O_2NCl$: N = 4.84 per cent.
Found: N = 4.88 per cent.

α,β-Isodiphenylchloroacetylaminoethanol and hexamethylenetetramine. The solution of the components in dry, boiling chloroform set to a thick cake after fifteen minutes. This was broken up and the boiling continued for one-half hour, after which the salt was filtered off and washed well with dry chloroform and dry acetone. An additional quantity was obtained by boiling the filtrate from the first crop. The salt is very difficultly soluble in water and gives a straw color with sulphuric acid. It melts and decomposes at 197–9°.

0.2317 gm. of substance required 5.28 cc. $AgNO_3$ Solution I.
Calculated for $C_{22}H_{28}O_2N_5Cl$: Cl = 8.25 per cent.
Found: Cl = 8.02 per cent.

[18] E. Erlenmeyer, Jr.: *Ann. d. chem.*, cccvii, p. 119, 1899; cccxxxvii, p. 320, 1904.

β-Phenyl-β-oxy-α-chloroacetylaminopropane,



92 grams of β-phenyl-β-oxy-α-chloropropane[19] were converted into the amino compound according to D. R.-P. 183361[20] and 189481,[14] except that the resulting mixture of bases was worked up by the usual methods instead of by the procedure given in the patents. Yield of β-phenyl-β-oxy-α-aminopropane: 28 grams, boiling at 133–40° at 14 mm. 14 grams of the amine were dissolved in benzene and chloroacetylated in the usual manner with chloroacetyl chloride in the presence of two-normal sodium hydroxide. Ether was added and the upper layer shaken out successively with 5 per cent aqueous hydrochloric acid, water, dilute sodium carbonate solution, and water. After drying over sodium sulphate the solvents were evaporated off and the hot residue was exhausted *in vacuo*. 15.7 grams of the chloroacetyl derivative were obtained as a viscous oil which could not be made to crystallize.

β-Phenyl-β-oxy-α-chloroacetylaminopropane and hexamethylenetetramine. The salt separates rapidly from the boiling chloroform solution of the components. It darkens slightly above 165° and melts with decomposition at 175–6°.

0.1972 gm. of substance required 9.96 cc. $AgNO_3$ Solution II.
Calculated for $C_{17}H_{26}O_2N_5Cl$: Cl = 9.64 per cent.
Found: Cl = 9.39 per cent.

α-Phenyl-α-benzoyloxy-β-benzoylaminopropane,

$$CH_3CH(NHCOC_6H_5)CH(OOCC_6H_5)C_6H_5.$$

In the course of a series of uncompleted experiments α-phenyl-α-oxy-β-aminopropane was prepared by the reduction of isonitrosopropiophenone with sodium amalgam. As the crude product so obtained melted 10 degrees below the melting point assigned

[19] M. Tiffeneau: *Compt. rend. Acad. d. sc.*, cxxxiv, p. 775, 1902.
[20] D. R.-P. 183361; *Friedlaenders Fortschr. d. Teerfarbenfabrikation*, viii, p. 1041, 1905–07.

by Rabe and Hunnius,[21] it was sought to identify the aminoalcohol by the preparation of some characteristic derivative. To this end 0.7 gram was dissolved in pyridine and treated in the cold with 3 mols. of benzoyl chloride. After standing for six hours the mixture was poured into water, precipitating an oil which soon solidified. After two recrystallizations from absolute alcohol, the dibenzoyl derivative formed minute, glistening prisms melting at 172.5–3.5° (corrected) with slight preliminary softening. Yield: 0.8 gram. It is readily soluble in acetone.

0.2972 gm. of substance (Kjeldahl) required 8.10 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{23}H_{21}O_3N$: N = 3.90 per cent.
Found: N = 3.82 per cent.

The chloroacetyl derivative of α-phenyl-α-oxy-β-aminopropane was prepared, but formed an uncrystallizable oil which decomposed partially when distilled in a high vacuum.

[21] P. Rabe and T. Hunnius: *Ber. d. deutsch. chem. Gesellsch.*, xlv, p. 2166 1912.

THE QUATERNARY SALTS OF HEXAMETHYLENE-TETRAMINE.

VI. HALOGENETHYL ETHERS AND ESTERS AND THEIR HEXAMETHYLENETETRAMINIUM SALTS.

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As far as the authors are aware, the literature contains no reference to the use of the halogenethyl ethers and esters for the preparation of quaternary salts of hexamethylenetetramine. In the present work the few chloroethyl compounds tested failed to react with the base, so recourse was had to the bromoethyl compounds. These were preferred to the iodo derivatives, since, as often observed with other types of halogen compounds, the salts resulting from the iodo derivatives proved to be insoluble in water.

With both the bromoethers and esters the reaction with hexamethylenetetramine was found to be very slow and in most cases the yields were poor. The best results were obtained by the use of a high boiling solvent such as toluene or by allowing the chloroform solution of the components to stand at room temperature for several months. The low solubility of hexamethylenetetramine in boiling toluene and the danger of decomposition at this high temperature are disadvantages of the former method.

During the work with this group of substances, occasion demanded the preparation of a number of new bromoethyl ethers and esters, a description of which will be found in the following.

EXPERIMENTAL.

(1) *Bromoethyl ethers and their derivatives.*

Phenoxyethylhexamethylenetetraminium bromide. The salt gradually separated in small yield from the solution of phenoxyethyl bromide and the base in dry chloroform during several hours' boiling. Additional amounts were obtained by further heating of the filtrate. The salt melts at 151–2°, is easily soluble in water, and gives a faint pink color with sulphuric acid.

0.2002 gm. of substance required 6.0 cc. $AgNO_3$ Solution I.[1]
Calculated for $C_{14}H_{21}ON_4Br$: Br = 23.42 per cent.
Found: Br = 23.78 per cent.

o-Methylphenoxyethylhexamethylenetetraminium bromide. 22 grams of *o*-cresoxyethyl bromide[2] and 15 grams of hexamethylenetetramine were dissolved in 150 cc. of dry chloroform and allowed to stand five months at room temperature. Yield: 30 grams of salt, melting at 185°. It forms glistening prisms and plates which dissolve with difficulty in water. It turns pink in sulphuric acid, giving an orange solution which gradually changes to red.

0.2091 gm. of substance required 5.9 cc. $AgNO_3$ Solution I.
Calculated for $C_{15}H_{23}ON_4Br$: Br = 22.49 per cent.
Found: Br = 22.38 per cent.

m-Methylphenoxyethyl bromide (m-cresoxyethyl bromide). 108 grams of *m*-cresol, a solution of 80 grams of 50 per cent aqueous sodium hydroxide in 250 cc. of absolute alcohol, and 300 grams of ethylene bromide were boiled three to four hours under a reflux. The mixture was then diluted with water, made distinctly alkaline with sodium hydroxide, and extracted with ether. The ether was distilled off and the residue fractionated *in vacuo.* The bromoethyl ether was obtained as a colorless oil boiling at 136–7° at 14 mm. Yield: 55 grams. This was redistilled before analysis.

0.1539 gm. of substance (Carius) gave 0.1314 gm. AgBr.
Calculated for $C_9H_{11}OBr$: Br = 37.16 per cent.
Found: Br = 36.34 per cent.

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[1] 1 cc. = 0.00352 gm. Cl; 0.00793 gm. Br; 0.01259 gm. I.
[2] L. Gattermann: *Ann. d. Chem.*, ccclvii, p. 356, 1907.

The high-boiling residue from the distillation of the above bromoethyl ether was taken up in hot alcohol and filtered. The ethylene ether separated on cooling, but was not further investigated.

m-Methylphenoxyethylhexamethylenetetraminium bromide. This was prepared in the same way as the *ortho* isomer. The salt separated with chloroform of crystallization, and was therefore boiled with dry acetone. As it was still found to be impure, it was taken up in hot absolute alcohol and filtered rapidly from the undissolved residue. 5 grams of the salt separated on cooling. It melted at 155–6°, dissolved readily in water, and gave a pink color with sulphuric acid.

0.1779 gm. of substance required 5.15 cc. $AgNO_3$ Solution I.
Calculated for $C_{15}H_{23}ON_4Br$: Br = 22.49 per cent.
Found: Br = 22.92 per cent.

p-Methylphenoxyethylhexamethylenetetraminium bromide. The salt was obtained in the same way as the *ortho* isomer. Yield: 33 grams, containing chloroform of crystallization. After boiling with dry acetone, the salt melted at 176°. It was somewhat more readily soluble in water than the *ortho* isomer, and gave no color with sulphuric acid.

0.2071 gm. of substance required 5.8 cc. $AgNO_3$ Solution I.
Calculated for $C_{15}H_{23}ON_4Br$: Br = 22.49 per cent.
Found: Br = 22.21 per cent.

α-Naphthyl bromoethyl ether (*α-naphthoxyethyl bromide*). This was prepared in the same way as *m*-cresoxyethyl bromide. Yield: 110 grams, boiling at 154–6° at 0.8 mm., from 145 grams of α-naphthol, 80 grams of 50 per cent aqueous sodium hydroxide in 250 cc. of absolute alcohol, and 500 grams of ethylene bromide. The boiling point was unchanged by a subsequent distillation. A few drops of the substance, on long standing in a freezing mixture, with occasional rubbing, yielded crystals which caused the solidification of the entire distillate. Recrystallized from ligroin with the aid of a freezing mixture, the ether forms transparent, hexagonal plates which melt at 25° (corrected) with slight preliminary softening. At room temperature it is sparingly soluble in methyl and ethyl alcohols, readily so in benzene, ether, and ligroin.

It turns orange under sulphuric acid and dissolves with a greenish yellow color.

0.2253 gm. of substance gave 0.1657 gm. AgBr.
Calculated for $C_{12}H_{11}OBr$: Br = 31.83 per cent.
Found: Br = 31.29 per cent.

Practically no ethylene ether was formed in this experiment.

α-Naphthoxyethylhexamethylenetetraminium bromide. This was prepared in the same manner as the three preceding salts. The compound forms glistening platelets which turn yellow above 170°, melt with decomposition at about 175°, and are very difficultly soluble in water. With sulphuric acid it gives a transitory green color which changes to brown by reflected light and blue by transmitted light.

0.2042 gm. of substance required 5.3 cc. $AgNO_3$ Solution I.
Calculated for $C_{18}H_{23}ON_4Br$: Br = 20.38 per cent.
Found: Br = 20.58 per cent.

β-Naphthoxyethylhexamethylenetetraminium bromide. The salt, prepared as in the above cases, separated in glistening leaflets containing chloroform of crystallization which could not be removed by boiling with acetone or alcohol. It is very difficultly soluble in water, gives an olive-yellow color with sulphuric acid, and decomposes at about 180°. An aqueous suspension, when boiled, evolves chloroform and acquires a nerolin-like odor.

0.2075 gm. of substance required 8.87 cc. $AgNO_3$ Solution II.[3]
Calculated for $C_{18}H_{23}ON_4Br \cdot \frac{1}{2}CHCl_3$: Br = 17.71 per cent.
Found: Br = 17.91 per cent.

2,4,6-Trichlorophenoxyethyl bromide. 50 grams of 2,4,6-trichlorophenol were dissolved in 100 cc. of absolute alcohol containing 20 grams of 50 per cent aqueous sodium hydroxide and mixed with 300 grams of ethylene bromide. The mixture was boiled for five hours, separating into two layers. After adding an excess of sodium hydroxide, the unused ethylene bromide was blown off with steam. On cooling, the oily reaction product crystallized. This was shaken out with chloroform, and, on evaporation of the solvent, remained in practically pure form.

[3] 1 cc. = 0.004192 gm. Br.

Recrystallized twice from absolute alcohol, it melts at 47–8° (corrected). It is readily soluble in ether, benzene, and toluene, difficultly in cold absolute alcohol.

0.1828 gm. of substance (Carius) gave 0.3707 gm. AgCl + AgBr.
Calculated for $C_8H_6OCl_3Br$: Cl = 34.93 per cent; Br = 26.24 per cent.
Found: Cl = 34.92 per cent; Br = 26.24 per cent.

2,4,6-Trichlorophenoxyethyldimethylamine hydrochloride. 20 grams of 33 per cent aqueous dimethylamine were added to a solution of 15 grams of trichlorophenoxyethyl bromide in 50 cc. of benzene. The flask containing the mixture was stoppered and frequently shaken during the course of three days, the aqueous layer, which at first floated on the benzene, gradually sinking to the bottom. The mixture was finally warmed for one hour on the water bath and then evaporated *in vacuo* to a syrup. This was taken up in water, made alkaline with sodium hydroxide, and extracted with ether. The residue obtained by concentration of the ether layer was treated with concentrated hydrochloric acid, diluted with absolute alcohol, and precipitated with ether. 7 grams of the hydrochloride were obtained in this way in the form of long, silky needles, melting at 187–9°. It dissolves readily in water and absolute alcohol.

0.1988 gm. of substance required 6.5 cc. $AgNO_3$ Solution I.
Calculated for $C_{10}H_{12}ONCl_3 \cdot HCl$: Cl^- = 11.62 per cent.
Found: Cl^- = 11.51 per cent.

2,4,6-Trichlorophenoxyethylpiperidine hydrochloride. 7 grams of piperidine were added to a solution of 10 grams of trichlorophenoxyethyl bromide in 25 cc. of benzene, after which the mixture was warmed on the water bath for two hours. The mixture was cooled, diluted with ether, and the piperidine hydrobromide filtered off and washed with ether. The filtrate was distilled to small bulk, made alkaline with sodium hydroxide, and freed from unchanged piperidine by distillation with steam, a small amount of the new base being also carried over. The residue was shaken out with ether, which was then dried and evaporated to small bulk. As the resulting oil did not crystallize, it was taken up in a little absolute alcohol, treated with an excess of concentrated hydrochloric acid, and evaporated on the water bath until crys-

tallization commenced. The mixture was then cooled and treated with ether until the maximum amount of hydrochloride had been precipitated. Yield: 7 grams. The salt melts at 188–9° and is easily soluble in water and alcohol.

0.2036 gm. of substance required 5.95 cc. $AgNO_3$ Solution I.
Calculated for $C_{13}H_{16}ONCl_3 \cdot HCl$: $Cl^- = 10.27$ per cent.
Found: $Cl^- = 10.28$ per cent.

p-Bromophenoxyethyl bromide. Phenoxyethyl bromide was treated in chloroform solution with 1.1 mols. of bromine. The solution was allowed to stand several hours and was then concentrated to a syrup. When this was chilled it formed a crystalline mass which was rubbed up with ligroin and sucked off. Recrystallized first from ligroin, then from absolute alcohol, it forms rhombs which melt at 56-7° (corrected) with slight preliminary softening. The bromide dissolves readily in benzene and ether, difficultly in cold absolute alcohol.

0.1466 gm. of substance (Carius) gave 0.1970 gm. AgBr.
Calculated for $C_8H_8OBr_2$: Br = 57.11 per cent.
Found: Br = 57.19 per cent.

p-Bromophenoxyethylhexamethylenetetraminium bromide. This was obtained by boiling the components in dry chloroform. The rate of addition was very slow. The salt decomposes at 185–6°, is difficultly soluble in water, and gives a faint yellow color with sulphuric acid.

0.2707 gm. substance required 6.57 cc. $AgNO_3$ Solution I.
Calculated for $C_{14}H_{20}ON_4Br_2$: Br = 19.03 per cent.
Found: Br = 19.25 per cent.

Tribromo-p-methylphenoxyethyl bromide (tribromo-p-cresoxyethyl bromide). 17 grams of tribromo-*p*-cresol, a solution of 4 grams of 50 per cent aqueous sodium hydroxide in 25 cc. of absolute alcohol, and 75 grams of ethylene bromide were boiled for four hours. The product was distilled with steam and the residue treated with alkali to dissolve the unchanged cresol. The undissolved oil was taken up in chloroform, dried, and evaporated to small bulk. The residue crystallized on cooling. Recrystallized from acetic acid, in which it is difficultly soluble in the cold, it

melts at 50–1° (corrected), with preliminary softening. It is easily soluble in benzene and acetone, less so in cold absolute alcohol.

0.1248 gm. of substance (Carius) gave 0.2090 gm. AgBr.
Calculated for $C_9H_8OBr_4$: Br = 70.77 per cent.
Found: Br = 71.30 per cent.

Tribromo-p-methylphenoxyethylhexamethylenetetraminium bromide. Equimolecular amounts of the components were allowed to stand in chloroform solution for four months. The deposited salt was filtered off, dried, and washed first with a little ice water, then with alcohol and ether. It melts at 181°, is very difficultly soluble in water, and gives no color with sulphuric acid.

0.1965 gm. of substance required 3.4 cc. $AgNO_3$ Solution I.
Calculated for $C_{15}H_{20}ON_4Br_4$: Br^- = 13.50 per cent.
Found: Br^- = 13.73 per cent.

Tribromo-p-methylphenoxyethylpiperidine hydrobromide. This salt was prepared for comparison with the hexamethylenetetraminium salt. 5 grams of tribromo-*p*-cresoxyethyl bromide were dissolved in 10 cc. of benzene and 2.5 grams of piperidine added. After boiling on the water bath for several hours, the precipitated piperidine hydrobromide was filtered off and washed with benzene. The filtrate was concentrated to a syrup, taken up in absolute alcohol, and treated with an excess of hydrobromic acid. The salt crystallized upon the addition of water, and was filtered off and washed with acetone and ether. The yield was almost quantitative. As the product had an irritating odor, it was recrystallized from a small amount of absolute alcohol. It is fairly readily soluble in water and is precipitated from its solution by hydrobromic acid. Heated in a tube sealed at both ends, it melts and decomposes at 223–30°.

0.2151 gm. of substance required 3.95 cc. $AgNO_3$ Solution I.
Calculated for $C_{14}H_{18}ONBr_3 \cdot HBr$: Br^- = 14.89 per cent.
Found: Br^- = 14.57 per cent.

Tetrabromo-p-methylphenoxyethyl bromide. In one experiment on the preparation of the tribromo compound in which an impure tribromo-*p*-cresol, containing apparently some tetrabromo cresol, had been used as starting material, a small amount of the tetra-

bromo compound was separated from the reaction product by virtue of its greater insolubility in absolute alcohol. It formed long, delicate, glistening needles, which, when recrystallized twice from acetic acid, melted at 106–7.5° with preliminary softening.

0.1632 gm. of substance (Carius) gave 0.2898 gm. AgBr.
Calculated for $C_9H_7OBr_5$: Br = 75.19 per cent.
Found: Br = 75.57 per cent.

o-Acetaminophenoxyethyl bromide. 75 grams of *o*-acetaminophenol were dissolved in a mixture of 40 grams of 50 per cent aqueous sodium hydroxide and 250 cc. of absolute alcohol. After adding 500 grams of ethylene bromide, the mixture was boiled for two hours. The alcohol and excess of ethylene bromide were distilled off with steam, after which the insoluble material solidified on cooling. This was dried and taken up in chloroform, in which the ethylene ether (30 to 40 grams) was found to be insoluble. The filtrate was evaporated to small bulk, precipitated with ligroin, and the product recrystallized from dilute alcohol. Yield: 60 grams. Recrystallized twice from absolute alcohol, the bromide forms needles melting at 90–0.5° (corrected) with preliminary softening. It is readily soluble in benzene and chloroform, less so in ether.

0.2769 gm. of substance (Kjeldahl) required 10.85 cc. $\frac{N}{10}$ HCl.
0.1806 gm. of substance gave 0.1330 gm. AgBr.
Calculated for $C_{10}H_{12}O_2NBr$: N = 5.43 per cent; Br = 30.96 per cent.
Found: N = 5.49 per cent; Br = 31.33 per cent.

o-Acetaminophenoxyethylhexamethylenetetraminium bromide. 17 grams of the salt were obtained from a mixture of 13 grams of bromide, 7 grams of hexamethylenetetramine, and 70 cc. of chloroform allowed to stand at room temperature for five months. The salt was further purified by boiling with dry acetone, after which it melted at about 160°. It is slowly, but freely, soluble in water and gives a faint pinkish color with sulphuric acid.

0.2069 gm. of substance required 5.15 cc. $AgNO_3$ Solution I.
Calculated for $C_{16}H_{24}O_2N_5Br$: Br = 20.09 per cent.
Found: Br = 19.74 per cent.

o-Aminophenoxyethyl bromide hydrobromide. *o*-Acetaminophenoxyethyl bromide was saponified by boiling for two hours with a mixture of one part of aqueous hydrobromic acid (d 1.49) and two parts of absolute alcohol. The amino hydrobromide, which separated in long needles on cooling, was recrystallized from absolute alcohol. It melts and decomposes at 193–8°. In aqueous solution it gives a lilac color with ferric chloride.

0.1997 gm. of substance gave 0.2543 gm. AgBr.
Calculated for $C_8H_{10}ONBr \cdot HBr$: Br = 53.83 per cent.
Found: Br = 54.20 per cent.

o-Aminophenoxyethyl bromide was obtained by adding just enough sodium hydroxide to a cold solution of the above salt. It separates as an oil which rapidly changes to glistening leaflets. These were quickly dried and recrystallized from ligroin, since, on keeping, the base liquefies owing to internal condensation. It melts at 36–7° (corrected).

0.2392 gm. of substance (Kjeldahl) required 11.05 cc. $\frac{N}{10}$ HCl.
Calculated for $C_8H_{10}ONBr$: N = 6.48 per cent.
Found: N = 6.47 per cent.

o-Aminophenoxyethylpiperidine hydrochloride. 15 grams of *o*-acetaminophenoxyethyl bromide in 100 cc. of benzene were treated with 12 grams of piperidine and warmed gently. The reaction proceeded smoothly, accompanied by the separation of piperidine hydrobromide. After standing over night, the mixture was heated for a short time on the water bath, cooled, treated with an equal volume of ether, and filtered. The filtrate was evaporated *in vacuo* to a syrup, taken up in absolute alcohol, and treated with an equal volume of concentrated hydrochloric acid. The mixture was boiled for two hours in order to remove the acetyl group, evaporated to small bulk *in vacuo*, taken up in absolute alcohol, and precipitated with ether. Yield: 15 grams. Recrystallized from absolute alcohol, the salt forms short, thick prisms melting at 184–5.5°. It dissolves readily in water and hot absolute alcohol, the former solution giving a deep brown color when warmed with a drop of ferric chloride solution.

0.2326 gm. of substance required 9.07 cc. $AgNO_3$ Solution I.
Calculated for $C_{13}H_{20}ON_2 \cdot HCl$: Cl = 13.82 per cent.
Found: Cl = 13.72 per cent.

o-Aminophenoxyethylpiperidine. In D. R.-P. 88502[4] it is stated that this substance is formed by the reduction of *o*-nitrophenoxyethylpiperidine, but no description of the product is given. As prepared by decomposition of the hydrochloride with sodium carbonate it forms a rapidly solidifying oil, which, recrystallized first from 50 per cent alcohol, then from ligroin, forms almost colorless diamond-shaped plates which melt at 69–70° (corrected) with preliminary softening. These are readily soluble at room temperature in the neutral organic solvents, excepting ligroin.

0.1243 gm. of substance gave 13.95 cc. N (774 mm. and 24°).
Calculated for $C_{13}H_{20}ON_2$: N = 12.72 per cent.
Found: N = 12.76 per cent.

p-Acetaminophenoxyethylhexamethylenetetraminium bromide. 13 grams of *p*-acetaminophenoxyethyl bromide, 7 grams of hexamethylenetetramine, and 70 cc. of chloroform were allowed to stand for five months at room temperature. 13 grams of the crude salt were obtained. After boiling out with dry acetone it melted at 196–8°. It is difficultly soluble in water and darkens in sulphuric acid, giving a faint blue-gray color.

0.1972 gm. of substance required 4.9 cc. $AgNO_3$ Solution I.
Calculated for $C_{16}H_{24}ON_5Br$: Br = 20.09 per cent.
Found: Br = 19.71 per cent.

o-Carbomethoxyphenoxyethyl bromide (2-bromoethoxybenzoic acid methyl ester). 50 grams of methyl salicylate, a solution of 28 grams of 50 per cent aqueous sodium hydroxide in 200 cc. of absolute alcohol, and 200 grams of ethylene bromide were boiled for four hours. The mixture was diluted with water, made alkaline, and extracted with ether. The residue obtained from the ether extract was fractionated *in vacuo*. The bromoethyl ether boils at 186–8° at 20 mm., and, on standing, partly solidifies. The crystalline portion was recrystallized twice from absolute alcohol, forming large, thick, transparent plates which melt at 37.5–8° (corrected). The compound is readily soluble at room temperature in the usual solvents except water. The yield was poor.

[4] *Friedlaenders Fortschr. d. Teerfarbenfabrikation*, iv, p. 814, 1894–97.

0.1741 gm. of substance (Carius) required 0.1239 gm. AgBr.
Calculated at $C_{10}H_{11}O_3Br$: Br = 30.85 per cent.
Found: Br = 30.30 per cent.

With hexamethylenetetramine in dry chloroform, the bromide gave only hexamethylenetetraminium bromide.

2-Bromoethoxybenzamide. 50 grams of salicylamide were dissolved in a solution of 8 grams of sodium in 250 cc. of absolute alcohol and 200 grams of ethylene bromide added. After boiling for one hour, the alcohol and excess of ethylene bromide were blown off with steam. The residual mixture was made alkaline and chilled, causing the gradual solidification of the oily reaction product. This was filtered off, boiled up with chloroform, allowed to cool, and the solution filtered from the residue of ethylene compound (14 grams; see below). The addition of ligroin to the filtrate resulted in the precipitation of the 2-bromoethoxybenzamide, which melted at 112–4°. Yield: 23 grams.

0.2449 gm. of substance (Kjeldahl) required 10.44 cc. $\frac{N}{10}$ HCl.
Calculated for $C_9H_{10}O_2NBr$: N = 5.74 per cent.
Found: N = 5.97 per cent.

Salicylamide ethylene ether,

$$o\text{-}H_2NOCC_6H_4OCH_2CH_2OC_6H_4CONH_2.$$

A portion of the ethylene ether obtained above was recrystallized twice from acetic acid and washed with acetone. It forms crystals which melt at 211.5° (corrected) and are difficultly soluble at room temperature in the usual solvents.

0.1948 gm. of substance (Kjeldahl) required 13.25 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{16}H_{16}O_4N_2$: N = 9.33 per cent.
Found: N = 9.53 per cent.

(2) Bromoethyl esters and their hexamethylenetetraminium salts.

Acetoxyethylhexamethylenetetraminium bromide. 9 grams of bromoethyl acetate (ethylene bromoacetin) and 7 grams of hexamethylenetetramine in 50 cc. of dry chloroform were heated in a sealed tube for one hour at 105°. The salt separated as silky needles which dissolved readily in water and melted at 172–3°.

The usual method of preparation resulted in the formation of an impure salt.

0.2058 gm. of substance required 6.95 cc. $AgNO_3$ Solution I.
Calculated for $C_{10}H_{19}O_2N_4Br$: Br = 26.03 per cent.
Found: Br = 26.79 per cent.

Benzoyloxyethylhexamethylenetetraminium bromide. 12 grams of bromoethyl benzoate[5] and 7 grams of hexamethylenetetramine were boiled for two hours in 200 cc. of dry toluene. The salt formed a thick mass of glistening needles on cooling. It melts to a yellow paste at 164–5° and is rather difficultly soluble in water.

0.2021 gm. of substance required 5.6 cc. $AgNO_3$ Solution I.
Calculated for $C_{15}H_{21}O_2N_4Br$: Br = 21.66 per cent.
Found: Br = 21.98 per cent.

Bromoethyl p-nitrobenzoate. 28 grams of ethylene bromohydrin and 40 grams of *p*-nitrobenzoyl chloride were heated on the water bath. Hydrochloric acid was slowly evolved, and the solution was finally heated in an oil bath at 140° until the evolution of gas ceased. The product, which solidified on cooling, was taken up in ether and shaken out with dilute alkali to remove *p*-nitrobenzoic acid. After concentration of the ether solution the ester solidified. Yield: 41 grams. Recrystallized twice from absolute alcohol, it forms faintly yellow rhombic crystals melting at 47–50° (corrected). It is readily soluble in benzene, chloroform, and ether.

0.1990 gm. of substance (Kjeldahl) required 6.90 cc. $\frac{N}{10}$ HCl.
0.1814 gm. of substance (Carius) gave 0.1192 gm. AgBr.
Calculated for $C_9H_8O_4NBr$: N = 5.11 per cent; Br = 29.16 per cent.
Found: N = 4.86 per cent; Br = 27.97 per cent.

p-Nitrobenzoyloxyethylhexamethylenetetraminium bromide. 10 grams of the bromoethyl ester and 5 grams of hexamethylenetetramine in 100 cc. of dry toluene were boiled for five hours. The liquid gradually became filled with a mass of glistening leaflets. 4 grams of the salt were obtained in this way, while the mother liquors yielded an additional quantity when boiled

[5] K. Auwers and E. Bergs: *Ann. d. Chem.*, cccxxxii, p. 209, 1904.

again. The compound melts with decomposition at 190–1° and is difficultly soluble in water.

0.1890 gm. of substance required 4.65 cc. $AgNO_3$ Solution I.
Calculated for $C_{15}H_{20}O_4N_5Br$: Br = 19.31 per cent.
Found: Br = 19.52 per cent.

p-Nitrobenzoyloxyethylhexamethylenetetraminium iodide. Before the above bromide had been prepared, an attempt had been made to combine chloroethyl *p*-nitrobenzoate with hexamethylenetetramine. As this had not been successful under the conditions tried, resort was had to the iodoethyl ester. This reacted very slowly with the base in boiling chloroform, precipitation of the salt being incomplete even after several days. The compound forms glistening yellow platelets which melt at 176–8° and are very difficultly soluble in cold water.

0.2017 gm. of substance required 4.45 cc. $AgNO_3$ Solution I.
Calculated for $C_{15}H_{20}O_4N_5I$: I = 27.53 per cent.
Found: I = 27.78 per cent.

During the present investigation the following bromoethyl esters were prepared, but because of pressure of other work no attempt was made to obtain the hexamethylenetetraminium salts from them.

Bromoethyl acetylsalicylate. To a well chilled solution of 10 grams of ethylene bromohydrin in 45 grams of pyridine were slowly added 16 cc. of acetylsalicylic chloride.[6] After several hours the mixture was poured into an excess of iced 25 per cent sulphuric acid. The red oil which separated was taken up in ether, washed with dilute sulphuric acid and water, dried over calcium chloride, and, after evaporation of the ether, fractionated in a high vacuum. The ester boils at 150–60° at 0.7 mm. and crystallizes on cooling. Recrystallized twice from absolute alcohol, it forms thick plates and prisms possessing an aromatic odor. It melts at 62–2.5° (corrected) with preliminary softening and dissolves readily in ether, chloroform, and benzene.

0.1575 gm. of substance (Carius) gave 0.1029 gm. AgBr.
Calculated for $C_{11}H_{11}O_4Br$: Br = 27.84 per cent.
Found: Br = 27.81 per cent.

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[6] R. Anschütz: *Ann. d. Chem.*, ccclxvii, p. 172, 1909.

Bromoethyl acetyl-p-cresotinate. The crude chloride prepared from 40 grams of acetyl-*p*-cresotinic acid[7] was dissolved in dry, alcohol-free ether and slowly added to a well chilled solution of 25 grams of ethylene bromohydrin in 125 grams of pyridine. After one-half hour the mixture was poured into water and the resulting heavy oil taken up in ether. This was shaken out successively with dilute sulphuric acid and sodium carbonate solution and dried. The residue obtained after evaporation of the ether crystallized on chilling and scratching, after which it was disintegrated with a little ligroin and sucked off. 25 grams of the ester were obtained after recrystallization from ligroin. Recrystallized from toluene with the aid of a freezing mixture, it forms delicate, glistening needles which melt at 62.5–3° (corrected) with slight preliminary softening. It is difficultly soluble in ligroin, easily in benzene and toluene.

0.1402 gm. of substance gave 0.0858 gm. AgBr.
0.1179 gm. of substance gave 0.2072 gm. CO_2 and 0.0458 gm. H_2O.
Calculated for $C_{12}H_{13}O_4Br$: C = 47.83%; H = 4.35%; Br = 26.54%.
Found: C = 47.92%; H = 4.35%; Br = 26.05%.

Bromoethyl anisate. 18 grams of ethylene bromohydrin and 24 grams of anisoyl chloride (from anisic acid and thionyl chloride) were warmed on the water bath. When the vigorous evolution of hydrochloric acid had ceased, water was added and the mixture shaken out with ether. This was washed with dilute sodium carbonate solution, dried, and evaporated to small bulk. The residue boiled at 189–91° at 14 mm., and yielded 27 grams of the ester. At 0.5 mm. the boiling point was 140° (corrected).

0.1915 gm. of substance required 14.05 cc. $AgNO_3$ Solution I.
Calculated for $C_{10}H_{11}O_3Br$: Br = 30.84 per cent.
Found: Br = 30.76 per cent.

Bromoethyl m-chloroacetylaminomethylbenzoate,

$$m\text{-}ClCH_2CONHCH_2C_6H_4COOCH_2CH_2Br.$$

15 grams of *m*-carboxychloroacetylbenzylamine (*m*-chloroacetylaminomethylbenzoic acid)[8] were converted into the chloride.[9]

[7] R. Anschütz and J. Sieben: *Ann. d. Chem.*, ccclxvii, p. 245, 1909.
[8] A. Einhorn and T. Mauermayer: *ibid.*, cccxliii, p. 295, 1905.
[9] See Paper II of this series, this *Journal*, xx, p. 693, 1915.

This was dissolved in chloroform and slowly added to a well chilled solution of 8 grams of ethylene bromohydrin in 50 grams of pyridine. After fifteen minutes the mixture was poured into an excess of iced 25 per cent sulphuric acid and shaken out with ether. When this was evaporated the residue crystallized. 20 grams of the ester were obtained after recrystallization from dilute alcohol. Recrystallized twice from benzene containing a little ligroin, then twice from absolute alcohol, using bone-black, it forms minute, pale yellow prisms which melt at 107–8.5° (corrected).

0.2010 gm. of substance (Kjeldahl) required 6.25 cc. $\frac{N}{10}$ HCl.
0.0806 gm. of substance gave 0.0780 gm. AgCl + AgBr.
Calculated for $C_{12}H_{13}O_3NClBr$: N = 4.19 %; Cl = 10.60 %; Br = 23.89%.
Found: N = 4.36 %; Cl = 10.36 %; Br = 23.35%.

THE QUATERNARY SALTS OF HEXAMETHYLENE-TETRAMINE.

VII. ω-HALOGEN DERIVATIVES OF ALIPHATIC-AROMATIC KETONES AND THEIR HEXAMETHYLENE-TETRAMINIUM SALTS.

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The preparation of quaternary salts by the addition of hexamethylenetetramine to halogen ketones has already been the subject of a study by Mannich and Hahn,[1] who used these substances as the starting material for the preparation of a few aminoketones according to the method of Delépine.[2] In our study of the quaternary salts of this base, we have had occasion to extend the work considerably in this direction. The results obtained will be the subject of the following.

Contrary to the facts observed in the use of the chloroacetyl derivatives of amino compounds, which, as a rule, reacted very smoothly with hexamethylenetetramine, the ω-chloroacetophenones were found to be unsuitable. This was due partly to the slowness of the reaction and partly to the decomposition which accompanied prolonged heating of the components. The best results were obtained by employing the bromo or iodoketones. These reacted very readily with the base and the products were easily isolated. The salts obtained from the bromo compounds were preferred to those derived from the iodides owing to their greater solubility.

In the course of the work, a number of new halogen ketones were prepared, following with slight variations the methods of Kunckell and his coworkers.[3]

[1] C. Mannich and F. H. Hahn: *Ber. d. deutsch. chem. Gesellsch.*, xliv, p. 1542, 1911.

[2] M. Delépine: *Bull. Soc. chim.*, series 3, xiii, p. 356, 1895; xvii, p. 293, 1897.

[3] F. Kunckell: *Ber. d. deutsch. chem. Gesellsch.*, xxx, pp. 577, 1713, 1897; xxxi, p. 169, 1898; xxxiii, pp. 2641, 2644, 1900.

EXPERIMENTAL.

ω-Bromoacetophenoneoxime and hexamethylenetetramine. 6.9 grams of ω-bromoacetophenoneoxime[4] were added to a warm solution of 4.6 grams of hexamethylenetetramine in dry chloroform. The reaction product precipitated immediately as a transparent gum. This gradually crystallized on standing several days with occasional rubbing. The crude salt thus obtained was ground up in a mortar with dry chloroform, filtered off, and washed well with the solvent. Yield: 11.2 grams. It melts with decomposition at 139°.

0.2040 gm. of substance required 6.05 cc. $AgNO_3$ Solution I.[5]
Calculated for $C_{14}H_{20}ON_5Br$: Br = 22.57 per cent.
Found: Br = 23.53 per cent.

p-Methylphenacylhexamethylenetetraminium bromide. 12 grams of *p*-methylphenacyl bromide (*p*-tolyl bromomethyl ketone[6]) were added to a solution of 8 grams of hexamethylenetetramine in dry chloroform. After the evolution of heat had ceased, the solution was boiled a few minutes, cooled, filtered from a slight precipitate, and treated with several volumes of dry acetone. The salt separated as slightly pinkish, minute prisms which melted at 148–9° to an orange-brown liquid. It dissolves readily in water and chloroform. Yield 13.9 grams.

0.2270 gm. of substance required 6.41 cc. $AgNO_3$ Solution I.
Calculated for $C_{15}H_{21}ON_4Br$: Br = 22.63 per cent.
Found: Br = 22.40 per cent.

p-Tolyl iodomethyl ketone (p-methylphenacyl iodide). 10 grams of *p*-tolyl chloromethyl ketone[6] were dissolved in a little dry acetone and treated with 70 cc. of a normal solution of sodium iodide in dry acetone. After standing several days in the dark at room temperature the mixture was poured into water, precipitating an oil which soon solidified. Recrystallized twice from 95 per cent alcohol, the iodide forms cream-colored needles which melt at 42–3° (corrected) and are readily soluble in the usual organic solvents. It has an acrid, irritating odor.

[4] H. Korten and R. Scholl: *Ber. d. deutsch. chem. Gesellsch.*, xxxiv, p. 1907, 1901.

[5] 1 cc. = 0.00352 gm. Cl; 0.00793 gm. Br; 0.01259 gm. I.

[6] Kunckell: *Ber. d. deutsch. chem. Gesellsch.*, xxx, pp. 577, 1713, 1897.

0.1463 gm. of substance (Carius) gave 0.1328 gm. AgI.
Calculated for C_9H_9OI: I = 48.82 per cent.
Found: I = 49.07 per cent.

p-Methylphenacylhexamethylenetetraminium iodide. On adding the iodo ketone to a cold solution of the base in dry chloroform heat was immediately evolved and the solution soon set to a solid cake. The salt separates practically quantitatively in the form of colorless micro-needles which melt at 157–8° to a red-brown tar. It is very difficultly soluble in cold water.

0.2761 gm. of substance required 6.93 cc. $AgNO_3$ Solution I.
Calculated for $C_{15}H_{21}ON_4I$: I = 31.72 per cent.
Found: I = 31.60 per cent.

o-Xylyl bromomethyl ketone. 11 grams of *o*-xylene were mixed with 22 grams of bromoacetyl bromide and added in small amounts to a suspension of 21.5 grams of powdered aluminium chloride in dry carbon disulphide. After the first vigorous evolution of hydrobromic acid had subsided the mixture was warmed slightly for about an hour and finally poured into ice water. The lower layer was taken up with ether, washed successively with dilute hydrochloric acid, dilute sodium carbonate solution, and finally with water. It was then dried, evaporated to small bulk, and diluted with ligroin before crystallization had set in. This modification of Kunckell's procedure was used throughout in preparing the alkyl-substituted chloro- and bromomethyl ketones used in the experiments described in this paper. By cooling the ligroin solution in a freezing mixture 21.5 grams of the *o*-xylyl bromomethyl ketone were obtained. Recrystallized from ligroin it forms large, slightly brownish needles with an irritating odor. It melts at 62–2.5° (corrected) and is somewhat less soluble in ligroin than in the other usual organic solvents. In sulphuric acid it gives a greenish yellow color, in alcoholic sodium hydroxide an orange color. It is probably identical with the product melting at 63–4° obtained by Armstrong and Kipping[7] by brominating *o*-xylyl methyl ketone, although these authors assign the bromine to a position on the aromatic nucleus.

0.1461 gm. of substance required 12.40 cc. $AgNO_3$ Solution II.[8]
Calculated for $C_{10}H_{11}OBr$: Br = 35.19 per cent.
Found: Br = 35.58 per cent.

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[7] H. E. Armstrong and F. S. Kipping: *Jour. Chem. Soc.*, lxiii, p. 86, 1893.
[8] 1 cc. = 0.004192 gm. Br.

o-Xylyl bromomethyl ketone and hexamethylenetetramine. This was prepared in the same way as *p*-methylphenacylhexamethylenetetraminium bromide. The crude product was purified by boiling for three-quarters of an hour with dry acetone. The salt melts at 137–8° to a red liquid.

0.1916 gm. of substance required 5.12 cc. $AgNO_3$ Solution I.
Calculated for $C_{16}H_{23}ON_4Br$: Br = 21.77 per cent.
Found: Br = 21.20 per cent.

m-Xylyl bromomethyl ketone. This was obtained in the same way as the *ortho* isomer. The yield was 11 grams. Recrystallized from ligroin with the aid of a freezing mixture, it forms colorless micro-leaflets with an irritating odor. It melts at 42–3° (corrected), is very soluble at room temperature in the usual organic solvents, and gives an olive-yellow color when dissolved in concentrated hydrochloric or sulphuric acid.

0.1428 gm. of substance gave 0.1188 gm. AgBr.
Calculated for $C_{10}H_{11}OBr$: Br = 35.19 per cent.
Found: Br = 35.41 per cent.

m-Xylyl bromomethyl ketone and hexamethylenetetramine. The addition of acetone to the filtered chloroform reaction mixture resulted in the deposition of the pure salt in the form of rosettes of needles. It melts at 145–6° to an orange-red liquid.

0.2141 gm. of substance required 5.77 cc. $AgNO_3$ Solution I.
Calculated for $C_{16}H_{23}ON_4Br$: Br = 21.77 per cent.
Found: Br = 21.38 per cent.

p-Ethylphenyl bromomethyl ketone (p-ethylphenacyl bromide). 8.3 grams of ethylbenzene (b p 131–5°) were mixed with 16 grams of bromoacetyl bromide and added in small amounts to a suspension of 16 grams of powdered aluminium chloride in dry carbon disulphide. The resulting mixture was worked up as in the other cases. Yield: 10.9 grams. The ketone forms glistening micro-platelets which melt at 33–4° (corrected) with slight preliminary softening and are very soluble in the organic solvents. It has a sharp odor, and gives a pale straw color with sulphuric acid.

0.1875 gm. of substance (Carius) gave 0.1595 gm. AgBr.
Calculated for $C_{10}H_{11}OBr$: Br = 35.19 per cent.
Found: Br = 36.20 per cent.

p-Ethylphenacylhexamethylenetetraminium bromide. When acetone is added to the filtered chloroform reaction mixture the salt gradually deposits as minute, glistening prisms. It melts and decomposes at 146–7°.

0.1794 gm. of substance required 4.90 cc. $AgNO_3$ Solution I.
Calculated for $C_{16}H_{23}ON_4Br$: Br = 21.77 per cent.
Found: Br = 21.67 per cent.

Mesityl bromomethyl ketone (2,4,6-trimethylphenacyl bromide). This substance, prepared as in the previous cases, forms glistening, colorless plates which melt at 55–6° (corrected) and are less soluble in ligroin than in the other organic solvents.

0.1475 gm. of substance (Carius) gave 0.1146 gm. AgBr.
Calculated for $C_{11}H_{13}OBr$: Br = 33.15 per cent.
Found: Br = 33.05 per cent.

m-Nitrophenacylhexamethylenetetraminium bromide. Equimolecular proportions of ω-bromo-*m*-nitroacetophenone and hexamethylenetetramine reacted immediately in warm, dry chloroform with separation of the crystalline salt. The mixture was heated to boiling for a few moments, after which it was allowed to cool and the substance filtered off. The salt was ground up with dry chloroform, filtered, and washed with dry acetone. It melts with decomposition at 157° and is difficultly soluble in the cold in water and absolute alcohol.

0.2246 gm. of substance required 10.86 cc. $AgNO_3$ Solution II.
Calculated for $C_{14}H_{18}O_3N_5Br$: Br = 20.77 per cent.
Found: Br = 20.27 per cent.

p-Acetaminophenyl bromomethyl ketone (p-acetaminophenacyl bromide). 30 grams of powdered aluminium chloride were added in small portions to a mixture of 12 grams of acetanilid, 30 grams of bromoacetyl bromide, and 70 cc. of dry carbon disulphide. After the reaction was completed the upper layer was poured off and the viscous, orange-red lower layer poured into ice water. The precipitate was filtered off and washed successively with dilute hydrochloric acid, water, and 50 per cent alcohol. Yield: 15.5 grams, after recrystallization from dilute alcohol or dilute acetic acid. Recrystallized from amyl alcohol, the ketone forms minute, cream-colored prisms which melt, when rapidly heated, at 190–3°

with decomposition. It is difficultly soluble in the cold in the common solvents.

0.3286 gm. of substance (Kjeldahl) required 13.05 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{10}H_{10}O_2NBr$: N = 5.47 per cent.
Found: N = 5.56 per cent.

p-Acetaminophenacylhexamethylenetetraminium bromide. Equal weights of the components were boiled in acetone for three hours. The salt separated as cream-colored prisms. These were filtered from the hot solution and washed well with acetone and chloroform. Yield: 75 per cent of the theory. When heated, the salt darkens and melts with decomposition at 194°. It is difficultly soluble in water.

0.2662 gm. of substance required 6.65 cc. $AgNO_3$ Solution I.
Calculated for $C_{16}H_{22}O_2N_5Br$: Br = 20.17 per cent.
Found: Br = 19.82 per cent.

p-Aminophenacylhexamethylenetetraminium chloride. 6.1 grams of hexamethylenetetramine and 7.4 grams of *p*-aminophenyl chloromethyl ketone[9] (*p*-aminophenacyl chloride) were boiled for about two hours in dry toluene. The process was accompanied by partial decomposition, but the light yellow microcrystalline salt that had separated was filtered off, boiled first with chloroform, then with toluene, and dried. Yield: 2.6 grams. The product as obtained did not dissolve completely in water.

0.1936 gm. of substance required 6.46 cc. $AgNO_3$ Solution I.
Calculated for $C_{14}H_{20}ON_5Cl$: Cl = 11.45 per cent.
Found: Cl = 11.75 per cent.

3-Acetamino-4-tolyl bromomethyl ketone (3-acetamino-4-methylphenacyl bromide). This substance was prepared from *o*-acettoluide in the same way as the *p*-acetaminophenacyl bromide. The yield was not quite as good. Recrystallized twice from acetic acid, then from acetone, the ketone forms pale drab-colored, felted needles melting at 167–70° with preliminary softening. The positions assigned to the substituting groups are the same as those proven for the corresponding chloroketone by Kunckell.[10]

[9] Kunckell: *Ber. d. deutsch. chem. Gesellsch.*, xxxiii, p. 2645, 1900.
[10] Kunckell: *Chem. Zentralbl.*, lxxxiii, pt. i, p. 136, 1912.

0.2448 gm. of substance (Kjeldahl) required 9.25 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{11}H_{12}O_2NBr$: N = 5.18 per cent.
Found: N = 5.29 per cent.

3-Acetamino-4-methylphenacylhexamethylenetetraminium bromide. On boiling the components in dry chloroform for one-half hour the salt was precipitated as a gum which finally settled to the bottom of the flask as a clear, brownish oil. This became crystalline after adding dry acetone and rubbing. The salt was filtered off, ground up, and washed well with dry acetone. It forms a pale brown, micro-crystalline powder which dissolves readily in water and decomposes at 150–5°.

0.1869 gm. of substance required 9.19 cc. $AgNO_3$ Solution II.
Calculated for $C_{17}H_{24}O_2N_5Br$: Br = 19.47 per cent.
Found: Br = 20.61 per cent.

3-Acetamino-4-tolyl ω-iodoethyl ketone (3-acetamino-4-methyl-ω-iodopropiophenone). 20 grams of powdered aluminium chloride were added in small portions to a mixture of 8.5 grams of *o*-acettoluide, 20 grams of β-iodopropionyl chloride,[11] and 50 cc. of dry carbon disulphide. At first clumps of an addition product separated. These gradually melted with the slow evolution of hydrochloric acid and the mixture separated into two layers. After standing for two days, the upper layer was discarded and the lower layer poured into ice water. The pale greenish yellow precipitate was washed with dilute hydrochloric acid, water, and a little 50 per cent alcohol, and recrystallized, with bone-blacking, from dilute alcohol. Yield: 12.8 grams. Recrystallized from toluene, it forms faintly greenish, felted needles which melt at 142–3° (corrected) to a dark liquid. It is difficultly soluble in the cold in chloroform, absolute alcohol, and toluene.

0.3796 gm. of substance (Kjeldahl) required 11.65 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{12}H_{14}O_2NI$: N = 4.23 per cent.
Found: N = 4.30 per cent.

In order to determine the positions of the substituting groups, 3 grams of the ketone were oxidized in acetone solution, with occasional cooling, with 5.5 grams of potassium permanganate. The filtrate from the manganese dioxide yielded, on acidification,

[11] See Paper III of this series, this *Journal*, xxi, p. 136, 1915.

a precipitate of the corresponding acid. 0.45 gram of this was obtained in the form of minute, glistening needles after recrystallization from acetic acid. It melts at 279–81° (corrected) and is therefore probably identical with the 3-acetamino-4-methylbenzoic acid [m. 267–70° (uncorrected)] obtained by Kunckell[10] by oxidizing 3-acetamino-4-tolyl chloromethyl ketone. After hydrolysis with hydrochloric acid it contains a diazotizable amino group.

0.2019 gm. of substance gave 12.8 cc. N (760 mm. and 22.5°).
Calculated for $C_{10}H_{11}O_3N$: N = 7.25 per cent.
Found: N = 7.13 per cent.

3-Acetamino-4-tolyl ω-iodoethyl ketone and hexamethylenetetramine. Equimolecular amounts of the components were warmed slightly in dry chloroform and allowed to stand over night. The resulting thick, crystalline mass was disintegrated and boiled for about ten minutes to complete the reaction. The salt was filtered off and warmed for two hours with dry acetone. When heated, it turns yellow at about 150° and melts at 161–2° with gas evolution. It is difficultly soluble in water.

0.2066 gm. of substance required 4.35 cc. $AgNO_3$ Solution I.
Calculated for $C_{18}H_{26}O_2N_5I$: I = 26.93 per cent.
Found: I = 26.52 per cent.

p-Methoxyphenacylhexamethylenetetraminium bromide. Instantaneous addition took place when *p*-anisyl bromomethyl ketone[12] was added to a solution of hexamethylenetetramine in warm, dry chloroform. After several hours about one-half volume of dry acetone was added and the salt filtered off and washed well with a mixture of two parts of dry chloroform to one part of dry acetone. The yield was practically quantitative. The salt forms faintly yellow micro-crystals which melt with decomposition at 172°. It is difficultly soluble in water and gives a bright yellow color with sulphuric acid.

0.2001 gm. of substance required 5.42 cc. $AgNO_3$ Solution I.
Calculated for $C_{15}H_{21}O_2N_4Br$: Br = 21.65 per cent.
Found: Br = 21.49 per cent.

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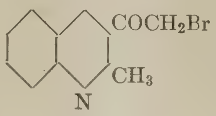
[12] F. Kunckell and W. Scheven: *Ber. d. deutsch. chem. Gesellsch.*, xxxi, p. 172, 1898.

p-Ethoxyphenacylhexamethylenetetraminium bromide. p-Phenetyl bromomethyl ketone[12] reacted vigorously with hexamethylenetetramine in warm, dry chloroform. The resulting crystalline cake was disintegrated, ground up with dry chloroform, and boiled with this for one-half hour. After adding an equal volume of dry acetone and allowing to stand for several hours, the product was filtered off and washed with chloroform-acetone and finally dry acetone. The salt forms slightly yellow micro-prisms which melt with decomposition at 159–9.5°. It is difficultly soluble in water, somewhat soluble in chloroform, and gives with sulphuric acid a yellow color changing to orange-brown.

0.1973 gm. of substance required 5.18 cc. $AgNO_3$ Solution I.
Calculated for $C_{16}H_{23}O_2N_4Br$: Br = 20.86 per cent.
Found: Br = 20.83 per cent.



(I)

β-[ω-bromoacetyl]-quinaldine (I). 25 grams of β-acetylquinaldine[13] were dissolved in 100 cc. of concentrated hydrobromic acid (d 1.49), warmed to 50° and rapidly turbined. To the solution were added, drop by drop, 25 grams of bromine dissolved in 50 cc. of hydrobromic acid.[14] During the course of the reaction a yellow hydrobromide of the bromoketone separated. After cooling, this was filtered off and washed with alcohol and ether. Yield: 35 grams. The free bromoketone was obtained from this by covering with water, in which it softens owing to dissociation, making alkaline with sodium carbonate, and shaking out with ether. The dried ethereal solution was evaporated to small bulk, leaving the bromoketone as an oil which solidified on chilling. Recrystallized first from ligroin, then toluene, then twice from methyl alcohol, it forms practically colorless rhombs which melt at 102–3° (corrected) with slight preliminary softening. It is readily soluble in ether and chloroform and slowly decomposes when heated above 50°.

[13] J. Eliasberg and P. Friedländer: *Ber. d. deutsch. chem. Gesellsch.*, xxv, p. 1756, 1892.

[14] According to the method of Kaufmann, *ibid.*, xlvi, p. 1831, 1913.

0.1380 gm. of substance gave 6.80 cc. N (764 mm. and 24.2°).
0.1510 gm. of substance gave 0.1081 gm. AgBr.
Calculated for $C_{12}H_{10}ONBr$: N = 5.30 per cent; Br = 30.26 per cent.
Found: N = 5.52 per cent; Br = 30.46 per cent.

β-[ω-bromoacetyl]-quinaldine and hexamethylenetetramine. Equimolecular amounts of the base and ketone (recrystallized once from ligroin) reacted almost instantaneously in dry, boiling chloroform, forming a solid, crystalline cake of the addition product. It melts with decomposition at about 170°. It is difficultly soluble in water, but easily so in 1 mol. of dilute hydrochloric acid.

0.2189 gm. of substance required 5.32 cc. $AgNO_3$ Solution I.
Calculated for $C_{18}H_{22}ON_5Br$: Br = 19.77 per cent.
Found: Br = 19.28 per cent.

THE QUATERNARY SALTS OF HEXAMETHYLENE-TETRAMINE.

VIII. MISCELLANEOUS SUBSTANCES CONTAINING ALIPHATICALLY BOUND HALOGEN AND THE HEXAMETHYLENETETRAMINIUM SALTS DERIVED THEREFROM.

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In this paper we wish to present a number of substances which could not be logically incorporated with the previous papers. These are derivatives of a number of simple alkyl halides and halogenacyl esters. Several chloroacetylamino compounds are also described.

EXPERIMENTAL.

(1) *Miscellaneous alkyl derivatives.*

Oxyethylhexamethylenetetraminium iodide. 1 mol. of iodoethyl alcohol was added to a solution of 1 mol. of hexamethylenetetramine in dry chloroform. The mixture was filtered from a small amount of precipitate that separated almost immediately, and allowed to stand for two days in a warm place. The salt crystallized in the form of needles. It melts at 157° with decomposition and is very soluble in water.

0.1622 gm. of substance required 5.25 cc. $AgNO_3$ Solution I.[1]
Calculated for $C_8H_{17}ON_4I$: I = 40.64 per cent.
Found: I = 40.74 per cent.

γ-Chloropropylhexamethylenetetraminium bromide. Trimethylene chlorobromide and hexamethylenetetramine reacted slowly in dry, boiling chloroform. Additional quantities of the quaternary salt may be obtained by continued boiling of the mother

[1] 1 cc. = 0.00352 gm. Cl; 0.00793 gm. Br; 0.01259 gm. I

liquors. The salt forms irregular micro-prisms which are very soluble in water. When rapidly heated, it turns yellow above 140° puffs up slightly, and finally melts with decomposition at 180–3°.

0.2456 gm. of substance required 8.23 cc. $AgNO_3$ Solution I.
Calculated for $C_9H_{18}N_4ClBr$: Br = 26.86 per cent.
Found: Br = 26.58 per cent.

γ-Oxypropylhexamethylenetetraminium iodide. This was prepared from 18.6 grams of trimethylene iodohydrin and 14 grams of hexamethylenetetramine in dry chloroform at room temperature. The portion (4 grams) that separated on standing over night was contaminated with hexamethylenetetraminium iodide, but the fraction (10 grams) separating during the next few days was free from this impurity.

0.2009 gm. of substance required 11.4 cc. $AgNO_3$ Solution II.[2]
Calculated for $C_9H_{19}ON_4I$: I = 38.92 per cent.
Found: I = 37.79 per cent.

Cetylhexamethylenetetraminium iodide. This was prepared in chloroform solution. The salt was recrystallized from absolute alcohol. It softens at 131° and melts gradually as the temperature is raised, liquefying completely and evolving gas at 157°. It is insoluble in cold water.

0.1940 gm. of substance required 7.58 cc. $AgNO_3$ Solution II.
Calculated for $C_{22}H_{45}N_4I$: I = 25.77 per cent.
Found: I = 26.00 per cent.

Phenylethyl iodide. Phenylethyl chloride, prepared according to Barger,[3] was treated with an excess of a normal solution of sodium iodide in dry acetone and boiled for four hours. The mixture, from which sodium chloride had separated, was poured into water and the precipitated oil taken up with ether. The ethereal solution was washed successively with dilute sodium bisulphite solution, water, sodium carbonate solution, and water. After drying over calcium chloride the ether was evaporated off. The residual oil was fractionated *in vacuo*, the portion boiling at 104.5–30.5° at 28 mm. being used directly for the preparation of the quaternary salt.

[2] 1 cc. = 0.00186 gm. Cl; 0.004192 gm. Br; 0.006657 gm. I.
[3] G. Barger: *Jour. Chem. Soc.*, xcv, p. 2194, 1909.

Phenylethylhexamethylenetetraminium iodide. The crude iodide reacted very slowly with hexamethylenetetramine in boiling chloroform. After several hours the mixture was diluted with more chloroform to dissolve unchanged base and filtered. An additional quantity was obtained by concentrating the filtrate and again boiling for several hours. The salt forms pale yellow cubes. When heated, it turns yellow and melts with decomposition at 159°.

0.2128 gm. of substance required 5.71 cc. $AgNO_3$ Solution I.
Calculated for $C_{14}H_{21}N_4I$: I = 34.11 per cent.
Found: I = 33.78 per cent.

The following substance was prepared since the publication of our first paper,[4] in which the compound properly belongs:

o-Iodobenzylhexamethylenetetraminium bromide. An equimolecular amount of *o*-iodobenzyl bromide was added to a warm solution of hexamethylenetetramine in dry chloroform. The salt soon began to crystallize in the form of spherical aggregates of minute crystals. After several hours these were washed well with dry chloroform and dry acetone and were then pulverized and warmed with dry acetone in order to remove traces of tenaciously adhering iodobenzyl bromide. The salt is sparingly soluble in water, but dissolves more readily than the *p*-isomer. It effervesces and turns yellow at 173–5°.

0.1822 gm. of substance (Kjeldahl) required 16.65 cc. $\frac{N}{10}$ HCl.
0.2767 gm. of substance required 11.52 cc. $AgNO_3$ Solution II.
Calculated for $C_{13}H_{18}N_4BrI$: N = 12.82 per cent; Br = 18.29 per cent.
Found: N = 12.80 per cent; Br = 17.45 per cent.

(2) *Esters of monohalogen acids.*

Carbethoxyethylhexamethylenetetraminium iodide. Equimolecular quantities of β-iodopropionic acid ethyl ester and hexamethylenetetramine were boiled in dry chloroform. The salt separated as a yellowish oil. This was seeded with a few crystals obtained by treating a portion with dry acetone and rubbing, causing solidification of the entire product. After continuing the boiling for one hour the precipitate of glistening

[4] This *Journal*, xx, p. 659, 1915.

leaflets was filtered off and washed thoroughly with dry chloroform. The salt dissolves readily in water and melts with decomposition at 137–8°.

0.2046 gm. of substance required 5.71 cc. $AgNO_3$ Solution I.
Calculated for $C_{11}H_{21}O_2N_4I$: I = 34.48 per cent.
Found: I = 35.14 per cent.

sec.-Octyl bromoacetate. 40 grams of bromoacetyl bromide were carefully added to 25 grams of *sec.*-octyl alcohol, with cooling. The mixture was then heated on the water bath until the evolution of hydrobromic acid ceased. The residue was fractionated *in vacuo*. The ester boils at 137° (corrected) at 18 mm. and possesses an odor resembling that of the alcohol.

0.1737 gm. of substance required 13.08 cc. $AgNO_3$ Solution II.
Calculated for $C_{10}H_{19}O_2Br$: Br = 31.83 per cent.
Found: Br = 31.58 per cent.

With hexamethylenetetramine this product yielded a gelatinous salt which could not be crystallized.

Bornyl bromoacetate and hexamethylenetetramine. Equimolecular amounts of the components were boiled in dry chloroform for one-half hour, filtered from the small amount of precipitated material, and treated with dry acetone. The solution gradually set to a thick mass of crystals of the salt. These were filtered off and washed with dry acetone. The compound sinters above 170° and melts at 178–81°. It is difficultly soluble in water.

0.2002 gm. of substance required 4.83 cc. $AgNO_3$ Solution I.
Calculated for $C_{18}H_{31}O_2N_4Br$: Br = 19.24 per cent.
Found: Br = 19.14 per cent.

Menthyl bromoacetate[5] *and hexamethylenetetramine.* The salt was prepared in the same way as the bornyl compound, using dry ether as the precipitant instead of acetone. It melts at 153–4° and is somewhat difficultly soluble in water.

0.2021 gm. of substance required 4.95 cc. $AgNO_3$ Solution I.
Calculated for $C_{18}H_{33}O_2N_4Br$: Br = 19.15 per cent.
Found: Br = 19.43 per cent.

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[5] S. Smiles: *Jour. Chem. Soc.*, lxxxvii, p. 454, 1905.

Phenyl bromoacetate and hexamethylenetetramine. Mannich and Drauzburg[6] prepared the corresponding salts with phenyl chloroacetate and phenyl iodoacetate. As we found the former to be unsuitable for preparation on a large scale and as the latter proved to be insoluble in water, the bromide was prepared. The quaternary salt separates rapidly from a boiling solution of the components in dry chloroform. As precipitated, it contains chloroform of crystallization which was removed by boiling the product with dry acetone. It melts at 149–50° with decomposition, and is easily soluble in water.

0.2262 gm. of substance required 6.40 cc. $AgNO_3$ Solution I.
Calculated for $C_{14}H_{19}O_2N_4Br$: Br = 22.51 per cent.
Found: Br = 22.45 per cent.

Tribromo-p-cresyl bromoacetate. Tribromo-*p*-cresol was dissolved in normal sodium hydroxide solution and treated, drop by drop, with chilling and shaking, with 1.1 mols. of bromoacetyl bromide. The oil which remained after making the mixture alkaline crystallized on rubbing. Recrystallized twice from absolute alcohol, it forms cream-colored prisms which soften at 60° and melt at 60.5–1.5° (corrected). The ester is more difficultly soluble in alcohol, methyl alcohol, and ligroin than in the other organic solvents.

0.1394 gm. of substance (Carius) gave 0.2247 gm. AgBr.
Calculated for $C_9H_6O_2Br_4$: Br = 68.64 per cent.
Found: Br = 68.60 per cent.

The hexamethylenetetraminium salt of the ester was found to be insoluble in water and was not obtained in a state of purity.

o-Nitrophenyl bromoacetate. This substance was prepared in the same way as the preceding ester. Recrystallized twice from absolute alcohol, in which it is less soluble than in ether, benzene, and chloroform, it melts at 55.5–6.° (corrected) with slight preliminary softening.

0.2794 gm. of substance (Kjeldahl) required 11.30 cc. $\frac{N}{10}$ HCl.
0.1777 gm. of substance gave 0.1270 gm. AgBr.
Calculated for $C_8H_6O_4NBr$: N = 5.39 per cent; Br = 30.74 per cent.
Found: N = 5.67 per cent; Br = 30.41 per cent.

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[6] C. Mannich and W. Drauzburg: *Chem. Zentralbl.*, lxxxiii, pt. ii, p. 1817, 1912.

o-Nitrophenyl bromoacetate and hexamethylenetetramine. The salt separates practically quantitatively when a solution of the components in dry chloroform is boiled for twenty minutes. It forms slightly grayish micro-prisms which melt with decomposition at 149–50°. Its aqueous solution decomposes rapidly with separation of *o*-nitrophenol. Moist air also exerts a hydrolytic action. The corresponding chloride was prepared by Mannich and Drauzburg.[6]

0.1216 gm. of substance required 3.06 cc. $AgNO_3$ Solution I.
Calculated for $C_{14}H_{18}O_4N_5Br$: Br = 19.97 per cent.
Found: Br = 19.96 per cent.

Benzeneazo-β-naphthyl chloroacetate. 4.2 cc. of chloroacetyl chloride were added to a solution of 12.4 grams of benzeneazo-β-naphthol in toluene. The mixture was boiled for nine hours and was then evaporated to small bulk. Separation of the ester was made more complete by the addition of ligroin. Yield: 6.7 grams. Recrystallized from absolute alcohol, it forms orange, felted needles which melt at 127–8° (corrected). It is very soluble in benzene, ether, and acetone, and dissolves in sulphuric acid with a purplish red color.

0.1310 gm. of substance gave 11.0 cc. N (750 mm. and 27.5°).
Calculated for $C_{18}H_{13}O_2N_2Cl$: N = 9.07 per cent.
Found: N = 8.63 per cent.

Oxyethyl anisate (oxyethyl p-methoxybenzoate). 6.2 grams of ethylene glycol were dissolved in 30 grams of pyridine and chilled in a freezing mixture. After slowly adding a solution of 17 grams of anisoyl chloride in 15 cc. of dry chloroform, the mixture was allowed to stand at room temperature for one hour. It was then poured into iced 25 per cent sulphuric acid and shaken out with chloroform. The oil remaining after concentration was fractionated *in vacuo*. 10 grams of the oxyethyl anisate distilled at 161–2° at 0.75–0.85 mm. When redistilled it boiled at 149–50° (corrected) at 0.4 mm.

0.1147 gm. of substance gave 0.2569 gm. CO_2 and 0.0624 gm. H_2O.
Calculated for $C_{10}H_{12}O_4$: C = 61.19 per cent; H = 6.17 per cent.
Found: C = 61.11 per cent; H = 6.09 per cent.

Ethylene anisate. The undistilled residue obtained in the fractionation of the oxyethyl anisate solidified on cooling. Recrystallized twice from acetic acid, it forms prisms which melt at 117–7.5° with slight preliminary softening. It is difficultly soluble in cold absolute alcohol and ether, easily in benzene and chloroform.

0.1161 gm. of substance gave 0.2787 gm. CO_2 and 0.0547 gm. H_2O.
Calculated for $C_{18}H_{18}O_6$: C = 65.41 per cent; H = 5.49 per cent.
Found: C = 65.48 per cent; H = 5.27 per cent.

Chloroacetyloxyethyl anisate. $ClCH_2COOCH_2CH_2OOCC_6H_4OCH_3$. A solution of 13.5 grams of crude oxyethyl anisate in 15 cc. of dry benzene was warmed with 7.5 grams of chloroacetyl chloride. After the evolution of hydrochloric acid had ceased the residue was fractionated *in vacuo.* The principal fraction boiled at 170–5° at 0.5 mm. and solidified on standing. Recrystallized successively from dry ether and absolute alcohol with the aid of a freezing mixture, it melts at 45.5–6.5° (corrected). It is very soluble in benzene and chloroform.

0.2822 gm. of substance required 19.39 cc. $AgNO_3$ Solution II.
Calculated for $C_{12}H_{13}O_5Cl$: Cl = 13.01 per cent.
Found: Cl = 12.75 per cent.

Chloroacetyloxyethyl anisate and hexamethylenetetramine. Equimolecular amounts of the components were boiled for one-half hour in dry chloroform, after which the solution was concentrated and filtered from a small amount of hexamethylenetetraminium chloride. The salt was then precipitated as a viscous oil by means of dry ether. The supernatant liquid was poured off and the oily salt dissolved in cold absolute alcohol. Ether was added until crystallization began, after which the mixture soon set to a solid mass. The salt was filtered off, washed, and placed in a desiccator as quickly as possible, owing to its hygroscopicity. When heated, it softens above 70° and gradually melts until it is completely fluid at 90°. It is easily soluble in water, alcohol, chloroform, and acetone.

0.1383 gm. of substance required 3.5 cc. $AgNO_3$ Solution I.
Calculated for $C_{18}H_{25}O_5N_4Cl$: Cl = 8.59 per cent.
Found: Cl = 8.91 per cent.

(3) Miscellaneous amine and hydrazine derivatives.

ω-Chloroacetylaminoacetophenone. 20 grams of ω-aminoacetophenone hydrochloride were dissolved in about 150 cc. of water and covered with about 100 cc. of toluene. After adding 11 cc. of chloroacetyl chloride, 150 cc. of two-normal potassium hydroxide were added in small amounts, with vigorous shaking and cooling. The chloroacetyl derivative soon separated and was filtered off, washed with water and toluene, and recrystallized, with bone-blacking, from dilute alcohol. Yield: 11.5 grams. It forms micaceous scales melting at 124–6.5° with preliminary softening. It is difficultly soluble in the cold in benzene, toluene, and ether, and dissolves very readily in chloroform.

0.2289 gm. of substance (Kjeldahl) required 11.15 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{10}H_{10}O_2NCl$: N = 6.62 per cent.
Found: N = 6.83 per cent.

ω-Chloroacetylaminoacetophenone and hexamethylenetetramine. A chloroform solution of the components was boiled for one-half hour, evaporated to small volume, filtered from the slight precipitate, and treated with dry acetone. The salt separated as an oil which rapidly solidified, forming micro-platelets with a faint yellow-green tinge. It melts at 149–50° to an orange tar, is soluble in chloroform, and dissolves readily in water and alcohol.

Calculated for $C_{16}H_{22}O_2N_5Cl$: Cl = 10.08 per cent.
Found: Cl = 10.00 per cent.

(I)

Chloroacetylbis-[p-dimethylaminophenyl]-methylamine (chloroacetylleucoauramine) (I). 8 grams of leucoauramine were dissolved in a mixture of benzene and toluene, 75 cc. of two-normal sodium hydroxide added, and then, drop by drop, 3 cc. of chloroacetyl chloride dissolved in toluene. Transitory colors developed, first green, then blue, then brown-violet, the color disappearing almost entirely in each case on shaking. Toward the end of the operation the chloroacetyl derivative started to crystallize

out. To make this complete one-half volume of ligroin was added and the mixture allowed to stand in the ice box over night. The product was filtered off, washed well with water, and recrystallized from 95 per cent alcohol, forming almost colorless plates and needles which take on a greenish blue tinge on exposure to the air. Yield: 7 grams. The compound dissolves in acetic acid with a blue color, in sulphuric acid with a faint yellow color, and is readily soluble in chloroform and acetone, but insoluble in ether. It melts to a deep green liquid at 164–4.5° (corrected) with slight gas evolution.

0.2442 gm. of substance (Kjeldahl) required 20.9 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{19}H_{24}ON_3Cl$: N = 12.16 per cent.
Found: N = 11.99 per cent.

Chloroacetylleucoauramine and hexamethylenetetramine. Equimolecular amounts of the components were boiled in dry chloroform solution for one and one-quarter hours. The olive-yellow solution was evaporated to small bulk and treated cautiously with dry acetone, causing the separation of the salt first as an oil. A portion of this was removed, rubbed up with acetone and ether, and crystallized by chilling. On seeding the main portion with this and slowly continuing the addition of dry acetone, the entire product was obtained in the crystalline state. After warming for one hour with dry acetone the salt still had a faint yellow color. It melts at 175° to a thick yellow liquid which decomposes at 179°. It is difficultly soluble in water but dissolves readily upon the addition of dilute acids.

0.3188 gm. of substance required 6.67 cc. $AgNO_3$ Solution I.
Calculated for $C_{25}H_{36}ON_7Cl$: Cl = 7.30 per cent.
Found: Cl = 7.36 per cent.

Chloroacetyltriphenylmethylamine (*chloroacettriphenylmethylamide*). 11.7 grams of crude triphenylmethylamine were obtained by passing dry ammonia into a mixture of 20 grams of triphenylmethyl chloride and 20 grams of naphthalene at 130°, taking up with ligroin, filtering off the ammonium chloride, saturating the filtrate with dry hydrochloric acid, suspending the resulting precipitate in dry benzene, passing in dry ammonia, again filtering, and evaporating the filtrate to dryness. The amine was

taken up in benzene and chloroacetylated as in the previous cases, completing the precipitation of the chloroacetyl derivative by means of ligroin. The compound was filtered off, washed with water, and recrystallized from acetic acid, forming snow-white, interlaced hairs. Yield: 5.9 grams. When recrystallized again from toluene and heated in a tube sealed at both ends it melts at 201–2.5° (corrected). The substance dissolves readily in chloroform, less easily in benzene and hot acetic acid, and with difficulty in absolute alcohol. It gives a yellow color with sulphuric acid, deepening to a dark orange-brown on addition of a drop of aqueous potassium dichromate solution.

0.3072 gm. of substance (Kjeldahl) required 9.35 cc. $\frac{N}{10}$ HCl.
Calculated for $C_{21}H_{18}ONCl$: N = 4.17 per cent.
Found: N = 4.26 per cent.

Chloroacetyltriphenylmethylamine and hexamethylenetetramine. After boiling for one hour the chloroform solution of the components was evaporated to small bulk and treated with several volumes of dry acetone. The precipitate of small, transparent cubes was boiled for one hour with dry acetone. When once isolated, the salt is difficultly soluble in chloroform. It also dissolves with difficulty in water and gives a yellow color with sulphuric acid. When heated it turns yellow at about 150° and melts with decomposition at 194–5°.

0.3173 gm. of substance required 6.27 cc. $AgNO_3$ Solution I.
Calculated for $C_{27}H_{30}ON_5Cl$: Cl = 7.46 per cent.
Found: Cl = 6.96 per cent.

β-Acetyl-α-chloroacetyl-α-phenylhydrazine and hexamethylenetetramine. The salt separated from the boiling chloroform solution of the components as long silky needles containing chloroform of crystallization. This was removed by boiling the compound for one-half hour with dry acetone. The salt melts at 192–3°.

0.1820 gm. of substance required 5.0 cc. $AgNO_3$ Solution I.
Calculated for $C_{16}H_{23}O_2N_6Cl$: Cl = 9.67 per cent.
Found: Cl = 9.67 per cent.

β-Chloroacetyl-α,α-phenylbenzylhydrazine. 20 grams of α,α-phenylbenzylhydrazine were chloroacetylated in toluene solution in the same way as in the previous examples. As the chloroacetyl

derivative did not separate from the toluene layer, the latter was shaken out first with dilute hydrochloric acid and then with dilute sodium carbonate solution. After the crystallization induced by the purification process had ended, the product was filtered off and combined later with an additional amount obtained by evaporating the filtrate to small bulk and adding ligroin. The substance was recrystallized by dissolving in hot benzene and adding an equal volume of ligroin. Yield: 17.8 grams. Recrystallized from methyl alcohol, it forms large, irregular crystals melting at 112.5–3.5° (corrected). It is readily soluble in the usual organic solvents, except ligroin.

0.1537 gm. of substance gave 14.25 cc. N (755 mm. and 17°).
Calculated for $C_{15}H_{15}ON_2Cl$: N = 10.21 per cent.
Found: N = 10.64 per cent.

β-Chloroacetyl-α, α-phenylbenzylhydrazine and hexamethylenetetramine. After the solution of the components in dry chloroform had been boiled for eight hours it was concentrated and treated with several volumes of dry acetone. The salt slowly crystallized out in almost quantitative yield. It melts at 145–6° to an orange liquid. It is readily soluble in water and dissolves in sulphuric acid with a brownish color.

0.2592 gm. of substance required 6.36 cc. $AgNO_3$ Solution I.
Calculated for $C_{21}H_{27}ON_6Cl$: Cl = 8.55 per cent.
Found: Cl = 8.64 per cent.

ON TISSUE FIBRINOLYSINS.

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If a small piece of tissue, with a diameter varying approximately between 1 and 3 mm., is taken from certain organs and placed in a test-tube containing three or four drops of blood plasma which has been kept liquid by the use of an oiled cannula, paraffined test-tube, and melting ice, the tissue coagulins present in the piece of tissue cause a more or less rapid coagulation of the plasma. The test-tubes are then placed in a thermostat, maintained at body temperature. The whole procedure, the withdrawal of the blood, the removal of the tissue, and the placing of the plasma and tissue into the previously sterilized test-tube, should be done with aseptic precautions.[1] The coagulum is at first closely attached to the piece of organ. If, however, we examine the pieces again after two or three hours, we find in the case of certain tissues a light zone between the tissue and coagulum. Sometimes this light zone, which is free from coagulum, surrounds the piece on all sides; at other times it is present around a part of the piece of tissue, while at other places the piece and coagulum still adhere to each other or are separated by a much narrower zone. After an interval of twelve to twenty-four hours the ring formation is frequently somewhat more pronounced, or in some cases may have appeared where it had not been visible previously. It is this phenomenon that we have analyzed.

[1] The principle of the procedure is the same as that for the cultivation of tissue *in vitro*, published by one of us in 1897. (L. Loeb: *Über die Entstehung von Bindegewebe, etc., und über eine Methode isolierte Gewebeteile zu züchten,* Chicago, 1897.) At that time coagulated blood serum, agar, and coagulated blood were used instead of coagulated blood plasma.

The dissolving effect of the organs of different species on various kinds of blood.

There are certain differences and also certain similarities between the organs of different species. The same organs have, on the whole, a tendency to cause a ring formation around the piece of tissue, while in others this power is almost or entirely lacking. In other respects, however, there exist marked differences between different species; not only are there differences in the efficiency of different organs within the same species and between organs of different species, but there are differences in the readiness with which the phenomenon can be obtained in the plasma of different species.

Table I shows the effect of guinea pig organs on rabbit, cat, and chicken plasma. If we consider the effect of the organs of the guinea pig on rabbit plasma, we may divide the various organs in an approximate way into three classes: (A), those which always or nearly always produce the ring formation, which we may provisionally interpret as due to a solution of the coagulum; (B), those which seldom or never have this effect; and (C), those in which the effect is more or less variable and usually not very pronounced. To class (A) belong the experimentally produced living deciduoma; the deciduoma which has become necrotic or has lost its power. The ovary, uterus, ureter, bladder, brain, sciatic nerve, stomach, duodenum, jejunum, mesentery, testicles, and thyroid were active in most cases, but there were a number of cases in which they were inactive. To class (B) belong the kidney, skin, and subcutaneous tissue of the guinea pig embryo and peritoneum. To class (C) belong the spleen, muscle, heart, liver, adrenal, subcutaneous connective tissue, pancreas, fat, and aorta. Lung tissue was usually negative, but in a few cases it was positive. The organs belonging to the genito-urinary system and some organs of the intestinal tract, mesentery, and nervous system are active. Urine we found inactive, as far as our method permitted us to determine. Pieces of spleen or muscle, which in themselves are inactive, were soaked in guinea pig urine and afterwards placed in plasma. Those pieces to which urine adhered had thereby not become active.

By far the most active organ, that which caused the greatest area of solution in the shortest time, was guinea pig ovary.

TABLE I.

	Rabbit Plasma		Chicken Plasma	Cat Plasma	
Guinea pig deciduoma	7+	6 o			
Guinea pig uterus	38+	–	14 o		
Rabbit uterus	22+	8 o	3 o		
Guinea pig kidney	25+	27 o	3 o		
Rabbit kidney	24+	39 o	4 o		
Guinea pig ureter	150+	8 o	10 o		
Rabbit ureter	157+	10 o	4 o	9+	–
Guinea pig spleen	3+	21 o			
Guinea pig spleen and urine	–	8 o			
Rabbit spleen	3+	43 o			
Rabbit spleen and urine	–	12 o			
Guinea pig muscle		18 o			
Guinea pig muscle and urine		4 o			
Rabbit muscle	4+	23 o			
Rabbit muscle and urine		4 o			
Guinea pig lung	3+	15 o			
Rabbit lung	2+	19 o			
Guinea pig testicle	18+	7 o			
Rabbit testicle	10+	13 o			
Guinea pig liver	3+	10 o			
Rabbit liver		16 o			
Guinea pig adrenal		5 o			
Rabbit adrenal		8 o			
Guinea pig connective tissue		2 o			
Rabbit connective tissue		8 o			
Guinea pig heart	1+	15 o			
Rabbit heart		16 o			
Guinea pig thyroid	21+	6 o			
Rabbit thyroid	3+	34 o			
Guinea pig pancreas	1+	12 o			
Rabbit pancreas	–	15 o			
Guinea pig brain	20+	–			
Rabbit brain	18+	4 o			
Guinea pig fat	–	8 o			
Rabbit fat	–	6 o			
Guinea pig sciatic	6+	–			
Rabbit sciatic	6+	8 o			
Guinea pig ovary	40+	–	10 o		
Rabbit ovary	2+	28 o	7 o		
Guinea pig jejunum	7+	–			
Rabbit jejunum	1+	6 o			
Guinea pig stomach	7+				

TABLE I—*Concluded.*

	RABBIT PLASMA		CHICKEN PLASMA	CAT PLASMA
Rabbit stomach	1+	5 o		
Guinea pig stomach and HCl	5+	4 o		
Rabbit stomach and HCl	1+	7 o		
Guinea pig duodenum	7+	–		
Rabbit duodenum	5+	3 o		
Guinea pig bladder	35+	3 o		
Rabbit bladder	30+	16 o		7+ 1 o
Guinea pig embryo skin	3+	2 o		
Guinea pig peritoneum	9+	9 o		
Guinea pig mesentery	9+	10 o		
Guinea pig aorta	–	18 o		

The figures with a plus sign indicate the number of effective, those with a negative sign, the number of ineffective pieces.

Guinea pig uterus was next. The bladder was usually more active than the ureter. The same organs in guinea pig plasma were much less active. This plasma is considerably less accessible to the dissolving power of guinea pig organs than rabbit plasma; but relatively the same order in the efficacy of the various organs is again brought out. Organs, like guinea pig ovary, which are always or almost always effective in rabbit plasma, are irregularly so in guinea pig plasma; while guinea pig brain, which is very active in rabbit plasma, is ineffective in guinea pig plasma. We find, furthermore, variations in the readiness with which guinea pig plasma can be dissolved; some coagula are more readily dissolved than others, and one may find a whole series of plasmas which are resistant to the action of organs. Where some solution of the coagulated plasma takes place, it is usually slight in comparison with the amount in rabbit plasma; but occasionally guinea pig plasma may show marked solution. Table II shows the differences in the solubility of guinea pig and rabbit plasma.

The various organs of the guinea pig are entirely ineffective towards coagulated chicken plasma.

Table I also shows the dissolving effect of rabbit organs on various plasmas. On the whole, rabbit organs behave in a manner similar to guinea pig organs on rabbit plasma. There are, however, some exceptions to this. The most striking is the almost

TABLE II.

Guinea pig ureter.........	Guinea pig plasma	16+	62 o
Guinea pig ureter.........	Rabbit plasma	62+	4 o
Guinea pig bladder.......	Guinea pig plasma	31+	43 o
Guinea pig bladder.......	Rabbit plasma	66+	1 o
Rabbit ureter..............	Guinea pig plasma	14+	65 o
Rabbit ureter..............	Rabbit plasma	59+	1 o
Rabbit bladder............	Guinea pig plasma	5+	58 o
Rabbit bladder............	Rabbit plasma	60+	15 o

total inefficiency of the rabbit ovary, which belongs to class (C), as compared with the guinea pig ovary, which was the most effective of all organs. Rabbit uterus still belongs to class (A), but is not quite as effective as guinea pig uterus. Rabbit testicle is less effective than that of the guinea pig, while the thyroid is almost ineffective (Class C). The sciatic nerve, jejunum, duodenum, and stomach are less active in the rabbit than in the guinea pig. Urine, tested as above, is ineffective.

Rabbit organs dissolve guinea pig plasma very much less than rabbit plasma. It is rare that a piece of rabbit organ dissolves guinea pig plasma, and solution is only brought about by those organs which act strongest on rabbit plasma. Towards chicken plasma rabbit organs are as ineffective as guinea pig organs. The ureter and bladder dissolve cat plasma, but the action is not as marked as with rabbit plasma.

The effects of cat organs on cat and on rabbit plasma are given in Table III. These organs are less efficient than guinea pig and rabbit organs. The same kind of organs is active in all three species, though in the cat the bladder and ureter are more active than the ovary and uterus. The bladder is especially active in rabbit plasma. This activity becomes apparent mainly on the second day. In general the effect in rabbit plasma is very slight on the first day, while on the second day it may become quite marked. The activity curve of the cat organs in rabbit plasma, as a function of the time, differs therefore from the curve of the guinea pig or rabbit organs. In cat plasma the dissolving effect of the cat organs becomes visible at an earlier time and does not usually progress much further on the following day. Cat liver and spleen are almost or entirely inactive.

TABLE III.

Cat organs.

	CAT PLASMA		RABBIT PLASMA	
Ureter	9+		18+	
Bladder	4+	4 o	15+	2 o
Testicle		4 o	4+	–
Uterus		4 o	9+	9 o
Ovary	3+	3 o	16+	1 o
Spleen	–		4+	15 o
Liver	–			12 o
Brain	–		4+	8 o
Kidney	–		2+	18 o
Lung	–		5+	5 o

Cat plasma coagulum is more resistant to solution than rabbit plasma, but more accessible than guinea pig plasma.

Mouse organs, tested with rabbit plasma coagulum, show the same sets of active organs. They are distinctly less active than guinea pig and rabbit organs and perhaps even somewhat less active than cat organs. As in the cat, lung is here somewhat more active than kidney, while the opposite is true in the guinea pig and rabbit. Mouse carcinoma was found, in the majority of cases, to be inactive; only in a few cases a very slight solution was observed.

TABLE IV.

Mouse organs.

	RABBIT PLASMA	
Tumor	4+	31 o
Uterus	7+	5 o
Ovary	5+	8 o
Bladder	5+	8 o
Spleen	1+	5 o
Liver	–	7 o
Lung	5+	5 o
Kidney	–	5 o

Chicken organs are entirely inactive, whether tested on chicken, rabbit, or guinea pig plasma. Even if the chicken plasma is diluted ten or fifteen times with distilled water no solution of the coagulum of the diluted plasma is noticeable.

TABLE V.

Chicken organs.

	CHICKEN PLASMA	RABBIT PLASMA	GUINEA PIG PLASMA
Ureter	18 o	4 o	3 o
Kidney	13 o	7 o	13 o
Uterus	15 o	4 o	8 o
Ovary	12 o	—	3 o
Testicle	3 o	3 o	—
Liver	10 o	3 o	—
Spleen	3 o	3 o	—
Lung	9 o	3 o	—
Heart	4 o	3 o	—
Muscle	3 o	3 o	—
Brain	3 o	3 o	—

In the chicken as well as in the rabbit and cat there is a certain parallelism in the power of the different species to dissolve coagulum and in the readiness with which the plasma can be attacked. The stronger the dissolving power of the organ of a certain species (with the exception of the guinea pig), the more readily the plasmatic coagulum of this species is dissolved.

Variations in the behavior of the organs and blood of different individuals of the same species.

Certain variations occur in the dissolving power of organs. At times the pieces of guinea pig organ are more active than the corresponding pieces of rabbit organ; the reverse may also occur. The relative efficacy of different organs (bladder and ureter) of the same animal also shows variations. There occur, furthermore, differences in the readiness with which the coagulated plasmas of different individuals of the same species can be dissolved. Thus there are individual variations not only in the strength of certain tissues but also in the character of the plasma of the same species.

The fact that different tissues behave differently towards the coagulum suggests that the different tissues of an organ might likewise differ in their dissolving power. The connective tissue of the subcutis was found to be inactive. From this it might follow that the connective tissues of other organs would also be

inactive. This would explain why, in the early stages of contact between pieces of organ and coagulum, solution takes place only on one side. In such cases a microscopical examination showed that the active side corresponded to the mucosa. In the case of the uterus, the epithelium of the mucosa was lacking, but the glands were present. In a similar case with a piece of ureter which had not been split open so that the mucosa had a chance to come in contact with the coagulum, examination indicated an eversion of the mucosa at the only place where solution had taken place. As controls, pieces of different kinds of nonliving material were tested. Pieces of glass and wood, with and without a coating of vaselin, were quite inactive. Pieces of paraffin were in most cases inactive; a slight retraction could be observed around two pieces. Substances upon which water does not readily spread out were purposely selected because these might be expected to show a tendency to allow retraction of the coagulum from their surfaces. This was, nevertheless, exceptional.

Does specificity exist in the dissolving effect of the organs of various species?

We have seen that both rabbit and guinea pig organs dissolve the coagulum of rabbit plasma and occasionally that of guinea pig plasma; cat and mouse organs also dissolve rabbit plasma. An absolute species specificity in the dissolving power of the various organs does, therefore, certainly not exist. Nevertheless, a specific adaptation between the organs and plasmas of various species may exist in the sense that organs of a certain species or class have a relatively stronger dissolving effect on the plasmatic coagulum of the same species or class than on the plasmatic coagulum of another species or class; such a specific adaptation has been demonstrated by one of us in the case of tissue coagulum.[2] Cross experiments were made, in which guinea pig and rabbit ureter and bladder were tested with guinea pig and rabbit plasmas (organs active in both species, and plasmas liable to be dissolved by these organs were chosen). Some cases were found in

[2] Loeb: *Medical News*, lxxxiii, p. 212, 1903; *Arch. f. Anat. u. Physiol.*, clxxvi, p. 10, 1904; *Biol. Bull.*, ix, p. 141, 1905.

which there seemed to be a relatively stronger action of the organs of one species on the plasma of the same species as compared with their action on the plasma of the other species. But in many experiments such a specific adaptation was lacking and the results do not, therefore, permit the conclusion that such a specific adaptation exists in the case of the fibrinolysins. It must be remembered, however, that even in the case of tissue coagulins, only a class and not a species specificity could be shown to exist in most cases.

The same conclusion was also reached as follows: In those cases in which pure rabbit plasma was more completely dissolved by rabbit organs (ureter and bladder) a mixture of equal parts of rabbit and guinea pig plasmas was dissolved as well or better by guinea pig organs than by rabbit organs. This is interpreted as indicating that guinea pig plasma is more accessible to solution by guinea pig than by rabbit organs, or that the dissolving power of guinea pig organs is less inhibited by guinea pig plasma than is that of rabbit organs. However, this effect was not constant and was never very marked.

The influence of heat on the dissolving power of organs.

Pieces of guinea pig ovary and ureter and of rabbit ureter, heated to 56° for thirty minutes, entirely lose their power to dissolve coagulum. This power is diminished (but not destroyed) by heating the pieces to 49° for thirty minutes. The number of ineffective pieces increased in the case of the rabbit and guinea pig ureter, and the effective pieces of rabbit ureter and guinea pig ovary were less active than the unheated control pieces.

TABLE VI.

The influence of heat on tissues in rabbit plasma.

	49° for 30 min.	50° for 30 min.	56° for 30 min.
Guinea pig ovary...	9+ (diminished)	3+ (diminished)	9 o
Guinea pig ureter..	7 o	—	7 o
Rabbit ureter.......	5+ (diminished) 3 o		9 o

The influence of temperature on the solution of coagulum by organs.

If the organs and plasma are kept at the temperature of melting ice instead of in the thermostat (39° to 40°) and examined after twenty-four hours, the solution of the coagulum is markedly less, and in the majority of cases is entirely lacking. Even after forty-eight hours it was still less than in the corresponding tubes at body temperature after twenty-four hours. Thus, at a temperature a few degrees above freezing, the solution of the coagulum is much retarded but not entirely prevented.

TABLE VII.

Pieces of organs in rabbit plasma kept on ice for twenty-four hours.

		DURING 1ST DAY	AFTER 48 HRS.
Guinea pig ovary...	10+ (markedly diminished) 2 o	4+ slight 8 o	4+ (1 marked, 3 slight)
Guinea pig uterus..	2+ (markedly diminished) 2 o	4 o	—
Guinea pig ureter...	— 4 o	4 o	—
Guinea pig bladder	1+ (markedly diminished) 3 o	1 slight 3 o	1 marked, 3 slight
Rabbit bladder.....	1+ (markedly diminished) 9 o	10 o	1 slight 3 o

The dissolving power of pieces of organ after a second transplantation into plasma.

It was of interest to determine whether the power of pieces of organs to dissolve coagulum was changed after they once had exerted this influence on coagulated plasma. Pieces of guinea pig ovary and bladder and rabbit bladder in coagulum were maintained near the freezing temperature for twenty-four hours and then transplanted into new plasma tubes, which were kept at body temperature for one day more. No noticeable loss in the dissolving power of the transplanted pieces was found. If, however, pieces of the same organs and, in addition, pieces of rabbit ureter in coagulum were left at 39° to 40° for twenty-four hours and then transferred to new tubes, the dissolving power of the pieces in the second set of tubes was very much diminished (Table VIII). Pieces of the same tissues, when kept in distilled

TABLE VIII.

Transplanted from rabbit plasma.

	ON ICE FOR 24 HRS.		IN THE THERMOSTAT FOR 24 HRS.
Guinea pig ovary.........	6 marked	1 questionable	3 o
Guinea pig bladder.......	4 marked		3 slight 2 o
Rabbit bladder...........	9 marked		5 medium 2 o
Rabbit ureter............	—		3 slight

water, in 0.85 per cent NaCl solution or in a dry test-tube, either at body temperature or on ice, lost in dissolving power when transplanted into plasma and kept at 39° to 40° for twenty-four hours (Table IX). In the latter case the washing out of the substance from the tissue or the drying of the tissues may have led to a loss in dissolving power.

TABLE IX.

	DRY		WATER		NaCl	
	Ice	Thermostat	Ice	Thermostat	Ice	Thermostat
Guinea pig ovary....	1 medium 1 slight	2 slight	1 slight 1 o	1 medium 1 o	1 medium 2 slight	2 o
Guinea pig uterus....	1 marked 1 o	2 marked	—	2 marked	1 medium 1 o	2 marked
Guinea pig ureter....	2 slight	2 medium	1 medium 1 o	2 o	1 o	2 slight
Rabbit bladder......	1 slight 1 o	2 slight 1 o	2 marked 1 medium	1 medium 1 slight	3 marked	3 slight
Guinea pig bladder..	1 marked	1 medium	1 slight	1 slight	1 medium	1 o

The influence of the contact with acid, alkali, glycerol, toluene, and fat-dissolving substances on the dissolving power of the organ.

In order to determine the effect of these substances, pieces of organs were left in contact with them for one day, then washed with sterile 0.85 per cent NaCl solution, and placed in the plasma. The pieces in alcohol, ether, and chloroform were kept at 0° and at room temperature; those in toluene, acid, alkali, and glycerol, at 0° and at 39° to 40°. The effect of these substances was not sufficient to render the pieces inactive, although a decrease in dissolving power took place. The temperature was not an important factor in the results.

TABLE X.

ORGANS LEFT 24 HRS. IN	OVARY	URETER	BLADDER
NaCl, toluene	1 o	3 slight 1 medium	3 marked, 1 medium, 2 slight 2o
N/50 HCl	1 slight 1 o		1 medium, 3 slight 4o
N/50 NaOH	2 slight		4 medium, 7 slight 5 o
Alcohol	—		1 marked, 4 medium, 3 slight 3 o
Ether	—		4 marked, 1 medium, 3 slight 3 o
Chloroform	—		4 medium, — 5 o
Glycerol	—		1 medium, 4 slight 3 o

The effect of the addition of acid, alkali, potassium cyanide, and Witte's peptone to the plasma on the dissolving power of the various organs.

In various experiments one drop of N/50 to N/10 hydrochloric acid or sodium hydroxide was added to two drops of plasma. To this mixture fresh pieces of organs were added. The addition of N/10 hydrochloric acid caused a distinct opacity in the plasma. In some tubes the plasma did not coagulate after the addition of the N/10 acid or alkali. The dissolving power of the guinea pig uterus was not noticeably changed by any of the substances used.

TABLE XI.

Influence of acid or alkali, 1 drop to 3 drops of rabbit plasma.

Guinea pig uterus	N/10 HCl 4+	N/20 HCl 2+	N/25 HCl 2+	N/50 HCl 7+
Guinea pig uterus	N/10 NaOH 6+	N/20 NaOH 3+	N/25 NaOH 3+	N/50 NaOH 8+

TABLE XII.

Influence of potassium cyanide, 1 drop to 2 drops of rabbit plasma.

Guinea pig ovary	N/10 1+	N/20 4+	N/25 7+	N/50 3+

TABLE XIII.

Influence of Witte's peptone, 1 drop to 3 drops of rabbit plasma.

Rabbit bladder	1% solution 6+	5% solution 7+

The effect of an atmosphere of hydrogen.

To determine the effect of hydrogen, a stream of this gas was passed through the tubes for about an hour after the pieces had been put into the plasma, and the hydrogen atmosphere was allowed to remain for a day. Solution of the coagulum proceeded in approximately the normal way.

TABLE XIV.

The influence of hydrogen on pieces in rabbit plasma.

Guinea pig ovary............	In plasma 7	On plasma 6
Guinea pig uterus...........	In plasma 7	On plasma 4 (2?)

The dissolving action of pieces of organs placed on the surface of coagulated plasma.

In the above experiments the plasma coagulated around the piece of organ. Therefore the pieces were at first in close contact with the coagulum. If active, an area gradually formed around the piece so that the coagulum was no longer in contact with it. There are two possible explanations for this phenomenon: first, it may be considered as a retraction of the coagulum from the various pieces; second, it may be assumed that certain pieces of organs actually have the power to dissolve coagulum in contact with them. In order to decide between the two possibilities, the plasma was allowed to coagulate at the bottom of the tubes before the pieces of organ were introduced. After standing for thirty to sixty minutes at room temperature, the tubes, each of which contained about four drops of plasma, had a coagulum sufficiently strong to permit the placing of a small piece of organ upon its surface. If the same differences among the various sets of organs were found in this case as were found by the first method, then it might be concluded that the phenomenon is due to a dissolving effect of certain organs on the coagulum rather than to a retraction of the coagulum from the pieces, although the latter may possibly, in certain cases, be added to the former. From Table XV it is seen that those organs, which are active when put into the liquid plasma, are about equally active when placed on the surface of the coagulated plasma. Likewise, the same kind of organs is inactive or slightly active in

both sets of experiments. While within the coagulum a light zone forms around the piece of organ, the active organs (as guinea pig ovary), when placed on the surface gradually dissolve the coagulum, sink into it (perhaps to the bottom of the tube), and then sometimes produce a light zone of dissolved coagulum around their circumference. With less active organs (rabbit or guinea pig bladder) solution often takes place, but is usually less complete. Organs such as liver, spleen, pancreas, or kidney, show the same activity (usually negative) when placed on the surface of the coagulum as when placed within the plasma. Pieces of glass, placed on the surface of the coagulum, produce a depression which disappears after their removal.

TABLE XV.

Tissues placed on the surface of coagulated rabbit plasma.

Guinea pig ovary.........	17 marked	4 medium	
Guinea pig placenta......	—	—	1 slight 2 o
Guinea pig bladder.......		9 medium	1 slight 1 o
Guinea pig uterus........	17 marked	—	— —
Guinea pig ureter.........	—	—	3 slight 1 o
Rabbit ureter.............	—	2 medium	— 2 o
Rabbit bladder...........	—	8 medium	6 slight 2 o
Guinea pig liver..........			14 o
Guinea pig kidney........			9 o
Guinea pig spleen.........			8 o
Guinea pig pancreas......			11 o
Glass.....................	(no permanent depression)		13 o

Can plasma coagulated by heating be dissolved by organs?

If the spontaneously clotted plasma is slowly heated to 72° or 85° and the pieces of organs are placed on it, no solution takes place, but the pieces remain on the surface of the plasma. The heat-coagulated plasma, therefore, can no longer be dissolved in a manner similar to the unchanged fibrinous coagulum.

TABLE XVI.

Tissues placed on the surface of heat coagulated rabbit plasma.

OVARY	UTERUS	PLACENTA	BLADDER	KIDNEY
8 o	10 o	3 o	5 o	6 o

Has the extract of active organs a dissolving effect?

An attempt was made to extract the fibrinolysins from organs which normally dissolve the coagulum, but it was unsuccessful. Finely divided guinea pig ovary or uterus or rabbit bladder was rubbed with a small amount of sterile 0.85 per cent NaCl solution and the suspension kept on ice or at room temperature for thirty to sixty minutes. One drop of the supernatant turbid fluid, added to plasma, did not produce a solution of the coagulum. The solid residue of the crushed ovary or uterus, when placed in the plasma, caused a slight solution, though this was not as marked as that produced by fresh pieces. The crushing of the piece probably removed or injured some of the active substance.

TABLE XVII.

Crushed organs in rabbit plasma.

	SUPERNATANT FLUID	RESIDUE
Guinea pig ovary	5 o	3 slight
Guinea pig uterus	4 o	—
Guinea pig spleen	4 o	—
Rabbit bladder	6 o	5 slight and slow 1 o

The effect of placing several pieces of tissue into the coagulum.

If instead of placing one single relatively large piece of organ in the plasma or on the surface of the coagulum, a large number of very small pieces of guinea pig ovary or uterus are distributed in the coagulum, a small zone of solution results around a number of the small pieces, or, as sometimes happens, the greater part of the coagulum is dissolved.

If the coagulum was covered with slices of ovarian tissue obtained by cutting the organ lengthwise into flat sections, the solution was superficial and not as complete as might have been expected. This may perhaps be due to the fact that only certain parts of the ovarian tissue have a dissolving effect, and those parts which had no dissolving effect (probably the medulla) prevented the other parts from sinking in and continuing their dissolving action.

The effect of the addition of various suspensions or emulsions to the plasma on the dissolving power of organs.

The observation that some organs are entirely without fibrinolytic power suggested the possibility that these organs contain substances which exert an inhibiting effect on the dissolving power of active organs. Emulsions (suspensions) of various organs in 0.85 per cent NaCl solution were prepared, with about two volumes of salt solution to one volume of organ, one drop of the emulsion was added to three drops of plasma, and both liquids were well mixed. The organ piece was added as usual. Liver emulsion had a very marked inhibiting influence on the dissolving power of guinea pig ovary and uterus. This effect was diminished, but not destroyed, by heating the emulsion to 56° for thirty minutes before adding it to the plasma. Spleen, kidney, mouse tumor, and brain emulsions had only a relatively slight inhibiting effect on fibrinolysis.

If instead of organ emulsion, one drop of rabbit serum was added to the plasma, no effect on the solution of the coagulum was observed.

Suspensions of charcoal and of kaolin were also prepared, one volume of the powdered substance being mixed with one and a half volumes of 0.85 per cent NaCl solution, and one drop of these was added to three drops of plasma. Kaolin had a slightly inhibiting effect, perhaps comparable with that exerted by kidney or brain. Charcoal was without any noticeable effect. This may be because charcoal had a tendency to collect at the bottom of the tube, only a little remaining in suspension in the coagulum surrounding the piece of organ. The margin of the coagulum adjoining the part between the coagulum and the piece of organ contained more charcoal particles than the other parts of the coagulum. This may indicate that some retraction of the border of the coagulum takes place in addition to the dissolving effect of the pieces of organs.

It may be concluded, therefore, that various suspensions of organs and also of inorganic material exert a certain inhibiting effect on the solution (or on the retraction) of the coagulum which takes place under the influence of the pieces of organs. This influence is probably not specific and may either be due to

the adsorption of the fibrinolytic agent by the admixed colloidal substances or to the changes in the structure and consistency of the coagulum which it produces. It seems, however, that the liver emulsion has in addition to this general action a special inhibitory effect on the fibrinolytic agency of certain organs. This assumption would be in harmony with the well known part which the liver plays in the regulation of the quantity of fibrinogen found in the blood. This interpretation of the action of liver emulsions is merely a tentative one.

TABLE XVIII.

The influence of tissue extracts upon ovary in rabbit plasma.

	MARKED	MEDIUM	SLIGHT	0
Control	24	3	6	2
Liver	1	—	3	—
Spleen	6	—	3	—
Kidney	4	6	2	—
Tumor	5	1	5	2
Brain	4	6	3	—
Liver at 56°	—	1	9	2
Charcoal	9	—	—	—
Kaolin	4	1	3	—
Serum	10	—	—	—

Influence upon uterus in rabbit plasma.

Control	1	1	3	—
Liver	—	—	2 (2?)	7
Brain	2	6	1	—

The effect of various pieces of organs on the dissolving power of guinea pig ovary.

The inhibiting effect of liver on the dissolving power of active organs, such as guinea pig ovary, can also be demonstrated in the following manner. When a small piece of liver is placed in plasma near the piece of ovary, but separated from the latter by coagulum, the liver exerts a distinct inhibiting influence on the dissolving power of the ovary. In most cases the dissolving power is not entirely destroyed, but is considerably diminished. The addition of pieces of spleen, mouse tumor, or glass instead of

liver, diminishes the dissolving action of the ovary little if at all. Boiling the pieces of liver before adding them to the plasma also destroys, more or less completely, their inhibiting power.

These experiments confirm the results obtained with other methods in which crushed material was added to the plasma. It may be assumed that substances exude from the liver into the surrounding plasma, which prevent the solution of the coagulum. The coagulum is stained around the pieces of liver and of spleen, thus indicating the admixture of the substances emanating from these organs into the plasma.

TABLE XIX.

The influence of two pieces of organs upon the ovary.

	MARKED	MEDIUM	SLIGHT	O
Control	7	2		
Liver	—	2	10	2
Spleen	10	4	2	—
Tumor	8	—	—	—
Glass	9	—	1	—
Boiled liver	6	2	3	—
Boiled tumor	5	1	—	—

Does chicken plasma contain a substance inhibiting the dissolving power of organs in rabbit coagulum?

Since an inhibiting substance has been demonstrated in the liver, it was thought possible that such an inhibiting substance might exist in the chicken plasma, since this cannot be dissolved by otherwise active organs. If this is so, we should expect to find that the addition of small amounts of chicken plasma exerts a definite inhibiting effect. This is, however, not the case. One part chicken plasma added to four parts rabbit plasma has no noticeable effect on the dissolving power of guinea pig ovary or uterus. If the amount of chicken plasma is increased, the number of active pieces of ovary or uterus gradually diminishes. It is probable that a soluble substance is lacking in chicken plasma; if sufficient chicken plasma is added to rabbit plasma, an insoluble part remains in the coagulum, which consists of the parti-

cles of chicken plasma intimately admixed with the rabbit plasma. There is no indication, therefore, that chicken plasma contains a substance which prevents the dissolving effect of otherwise active organs on rabbit plasma.

TABLE XX.

The influence of chicken plasma.

	OVARY	UTERUS
1 part chicken plasma plus 4 parts rabbit plasma.....	3+	2+
1 part chicken plasma plus 3 parts rabbit plasma.....	5+ 1 o	4+ 2 o
1 part chicken plasma plus 2 parts rabbit plasma.....	3+ 3 o	2+ 4 o

If equal parts of rabbit and guinea pig plasma are mixed and guinea pig or rabbit organs added, the dissolving power of the organs in the mixture is, on the whole, greater than that in pure guinea pig and less than that in rabbit plasma. In a few cases the solution was greater in the mixture than in rabbit plasma, especially if guinea pig organs were used.

TABLE XXI.

Comparison between the action of various organs in rabbit plasma and in a mixture of rabbit and guinea pig plasmas.

	RABBIT URETER	GUINEA PIG URETER	RABBIT BLADDER	GUINEA PIG BLADDER
More marked in rabbit plasma....................	6	8	10	6
More marked or equal in rabbit plasma................	2	2	2	2
Equal in both................	1	—	1	1
More marked or equal in mixture..................	—	—	—	3
More marked in mixture.....	2	1 (questionable)	1 (slight)	2

In all cases the organs are less active in guinea pig plasma than in the mixture (excluding the cases where the organs were negative in both).

Does a relationship exist between the dissolving power of various organs and their effect on the coagulation of the blood?[3]

The fact that some of the organs which are most active in accelerating the coagulation of the blood, like the spleen and the liver, and to some extent the kidney, are least active in dissolving coagulum, suggested a possible relationship between the two factors. Experiments were therefore carried out in which comparative tests were made of the effect of these organs upon the coagulation of the blood. Small pieces of various organs were placed at the bottom of small test-tubes and three drops of rabbit plasma, containing hirudin, added. Table XXII gives the averages of the coagulation time in the various experiments. List No. II differs from No. I in that one experiment, in which a stronger solution of hirudin was used, is omitted. From these results it may be concluded that there exists a certain relationship between the fibrinolytic power of organs and their effect on the coagulation of the blood, but that other factors intervene. Guinea pig ovary and uterus, which have the strongest fibrinolytic effect, have the weakest effect in accelerating the coagulation of the blood. Bladder and ureter, which have fairly marked fibrinolytic power, also accelerate the coagulation of the blood fairly markedly. These results may be explained by assuming that nearly all organs contain tissue coagulins or other factors which accelerate the coagulation of the blood, while certain of these organs contain, in addition, fibrinolytic substances which tend to prevent the coagulation. Bladder and ureter probably contain relatively large quantities of those factors which favor the coagulation of the blood.

[3] We take this occasion to point out a probable error in a former publication concerning the coagulation of the blood (M. Vera and L. Loeb: this *Journal*, xix, p. 305, 1914). We concluded that immunization with hirudin changed the reaction of the blood towards sodium fluoride in a similar way as towards hirudin, though less markedly. Our evidence was however contradictory in as far as experiments in which we used the blood-serum of the immunized animals were negative; in this case sodium fluoride behaved like other substances preventing coagulation of the blood through inactivation of calcium. We made therefore additional experiments with sodium fluoride and found that it behaved in all cases like citrate and oxalate or magnesium chloride. We intend to take up this question again as soon as an opportunity presents itself.

Experiments carried out without the previous addition of hirudin to the plasma, in which the plasma was kept a few degrees above the freezing point during the progress of the work, gave similar results.

Experiments in which organ suspensions were added to the plasma are of interest inasmuch as they demonstrate the strong accelerating effect exerted by the brain tissue (lipoids) under these conditions.

TABLE XXII.

Coagulation time.

I. One drop of hirudin solution (0.05 to 0.03 per cent) and three drops of rabbit plasma.

Five experiments.

GUINEA PIG OVARY	GUINEA PIG UTERUS	RABBIT OVARY	GUINEA PIG PANCREAS	GUINEA PIG LUNG	GUINEA PIG URETER	GUINEA PIG BLADDER	GUINEA PIG SPLEEN
min.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
38.2	28.6	17	15.4	14	12.6	11.4	8.2

II. One drop of hirudin solution (0.04 to 0.03 per cent) and three drops of rabbit plasma.

Four experiments.

CONTROL	GUINEA PIG OVARY	GUINEA PIG UTERUS	GUINEA PIG UTERUS (CRUSHED)	GUINEA PIG PANCREAS	GUINEA PIG BRAIN (2 EXPERIMENTS)
min.	*min.*	*min.*	*min.*	*min.*	*min.*
31.5	28	16	13	12.5	10.75

LUNG	LIVER	URETER	BLADDER	KIDNEY	RABBIT OVARY	SPLEEN
min.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
9.75	9.25	9	8.75	8.5	8.25	6.25

CONCLUSIONS.

The above observations make it appear probable that there exist in various species of animals certain tissue substances which have the power to dissolve blood coagulum when it is in direct contact with the tissues. These substances may be called fibrino-

lysins.[4] They seem to diffuse slowly out of the cells and to act only in the environment of the tissues. Heating the tissues to 49° or 50° weakens their activity, while heating to 56° destroys it. This action is probably due to a direct, injurious effect on the fibrinolytic agency, although we cannot entirely exclude the fact that the coagulating effect on the tissues, which prevents the active agent's escaping from the tissue, may also be concerned in it. The fibrinolytic efficiency of the organs of different species varies considerably. Some, like chicken organs, seem entirely inactive. Similar differences appear in the readiness with which various kinds of blood are dissolved. Here, again, bird blood has been found to be insoluble. There seems to be a certain, although not complete, parallelism between the fibrinolytic power of the organs of a certain species and the readiness with which its blood is dissolved, although further investigation is needed to establish this point.

In the species thus far examined, the same kinds of organs were found to be active. The genito-urinary system is the most active, while the nervous system and certain organs of the alimentary tract are next. Other organs, as spleen, liver, pancreas, muscle, heart, connective tissue, and mouse tumor, have been found to be nearly or entirely inactive. There are certain variations from this general statement. The most striking difference, perhaps, is between the ovary of the guinea pig and that of the rabbit. While the former is the most active organ found thus far, the latter is almost inactive. Not all the cells contributing to the building of an organ are concerned in its activity; ordinarily connective tissue seems to be inactive.

The work also indicates that there is a specific adaptation between the fibrinolytic action of the organs of a certain species and its own blood. This follows from the fact that the addition of guinea pig blood to rabbit blood, which is much more soluble than guinea pig blood, has apparently a smaller inhibiting effect

[4] H. Conradi (Über die Beziehungen der Autolyse zur Blutgerrinnung, *Beitr. z. chem. Phys. u. Path.*, i, p. 136, 1902) mentions briefly that the press juice from liver, spleen, and testicle has a fibrinolytic action. He does not, to our knowledge, mention any detailed results. Liver and spleen are, in our experience, the organs that do not show fibrinolytic action.

on the solution of the coagulum by guinea pig organs than upon that by rabbit organs. However, further proof is needed before definitely deciding the question. The fact that placing the organs on the coagulum has as definite an effect as putting the pieces into the coagulum makes it appear certain that the phenomena cannot be explained as caused by mere retraction of the coagulum, although this may be a secondary factor.

The active substance has not yet been extracted from the crushed pieces of organs with normal salt solution, nor has an emulsion of brain mixed with blood been found active, although pieces of brain often show a certain degree of activity.

In interpreting this latter observation one has to consider the retarding influence which certain powders like kaolin and organ suspensions exert on fibrinolysis and perhaps also on the retraction of the coagulum. It is possible that antagonistic factors (adsorption) or the presence of antagonistic substances in certain organs complicate the experiments, and that in finely divided condition the antagonistic factors overbalance the fibrinolytic power. In most organ suspensions this inhibiting effect is relatively slight. The most marked effect has been found in liver suspensions. This inhibiting power of the liver can also be demonstrated if a small piece of liver is placed near a piece of guinea pig ovary or uterus. A substance diffuses from the liver into the coagulum which inhibits, to some extent, the fibrinolytic power of the ovary or uterus. It is interesting to consider in this connection that the liver is especially the organ that regulates fibrinolysis in the blood, and that after exclusion of the liver from the circulation fibrinolytic processes are much accelerated in the blood.

Thus while certain organs which in themselves do not exert a fibrinolytic activity can be shown to contain an antagonistic factor, this does not seem to be so in the blood of those species which are insoluble. Chicken blood does not seem to prevent the solution of rabbit blood if added in small quantity, but seems merely to lack a soluble substance; if both bloods are mixed in equal parts the chicken blood provides an insoluble structure between which the soluble rabbit particles are situated. Some of the organs which have the greatest fibrinolytic power, like the guinea pig ovary and uterus, have the smallest power of acceler-

ating the coagulation of blood. Rabbit ovary, which has very slight fibrinolytic power, is less unfavorable to the coagulation, but still does not accelerate it markedly. On the other hand, some of those organs, as spleen, liver, or kidney, which have an irregular or no fibrinolytic effect, accelerate the coagulation of the blood markedly. This suggests that there is a relation between the power to influence the coagulation of blood and the fibrinolytic effect.

Schickele[5] and quite recently Fujii[6] found that the ovaries and uterus contain substances inhibiting the coagulation of the blood. Fujii found that the rabbit uterus is more active than the ovary, while in other animals the ovary is more active. Correspondingly, we have found that the ovary of the rabbit is less active than that of some other species. Fujii also found that the placenta contained substances inhibiting the coagulation of blood, a fact which agrees with the above demonstration of the fibrinolytic power of the placenta. These facts suggest that the substance inhibiting the coagulation of the blood is identical with the fibrinolytic substance. Such an assumption would be in accord with the observation previously published by one[7] of us; namely, that in phosphorus poisoning there exists a parallelism between the decrease of the fibrinogen and of the substances of the blood which accelerate its coagulation. The present investigation also suggests that the presence of this substance is not the only factor determining the fibrinolytic power of organs, but that there may be agencies at work in various organs counterbalancing the fibrinolytic effect of the active organs.

The fibrinolysins contained in certain organs, and absent or masked in others, are of practical significance in pathological conditions. This significance lies especially in two directions. First, the ingrowth of connective tissue and other cells into certain areas depends, as has been previously shown, upon the sensitiveness to stereotropic stimuli on the part of the cells which organize defects of various kinds.[8] Contact of cells with coagulum is, there-

[5] G. Schickele: *Biochem. Ztschr.*, xxxviii, p. 169, 1912.

[6] T. Fujii: *ibid.*, lxvi, p. 368, 1914.

[7] Loeb: *Beitr. z. chem. Phys. u. Path.*, v, p. 534, 1904.

[8] Loeb: Ueber Regeneration des Epithels, *Arch. f. Entwcklngsmechn. d. Organ.*, vi, p. 297, 1898.

fore, an essential factor in determining the movements of the cells. The tissue coagulins present in many or perhaps all organs tend to establish such a contact and therefore promote organization of various defects, of thrombi or of coagulated transudates.[9] Tissue fibrinolysins present in some organs have the opposite tendency; they prevent the ingrowth of cells. Thus, when pieces of guinea pig ovary or uterus are placed in blood plasma, contact between the tissue and the solid coagulum is lacking and outgrowth of cells into the surrounding fluid does not take place. This is in accordance with the presence of fibrinolysins in these organs. Second, it may be that the formation of cysts filled with liquids containing remnants of blood, which develop after hemorrhage in the brain, and perhaps also in other places, depends upon the action of tissue fibrinolysins, which gradually liquefy the coagulum.

The tissue fibrinolysins are, as far as can be determined at the present time, not identical with those tissue-splitting ferments which produce autolysis. The latter are, according to Jacoby, specific; they digest only their own organ. They are, furthermore, strongest in those organs, as the liver, in which we could not detect fibrinolysins.

SUMMARY.

Certain organ systems, especially the genito-urinary and nervous systems, contain fibrinolysins. Heating organs to 56° for thirty minutes renders them inactive. Various species of animals differ in the fibrinolytic power of their organs; there also exist great variations in the solubility of different kinds of blood. The liver counteracts the effect of the fibrinolysins. The substances found in certain organs, which inhibit the coagulation of the blood, are perhaps identical with the fibrinolysins. The fibrinolysins have a considerable biological significance in preventing growth processes; they are antagonistic to the tissue coagulins which promote growth processes. The fibrinolysins do not seem to be identical with those splitting ferments which produce autolysis.

[9] Loeb: On the coagulation of the blood in its relation to thrombosis and the formation of fibrinous exudate, *Montreal Med. Jour.*, xxxii, p. 507, 1903.

ON THE NATURE OF THE SUGARS FOUND IN THE TUBERS OF SWEET POTATOES.

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This is a second paper on the results of investigations, now being conducted in our laboratory, concerning the sugars in the underground reserve organs of plants.[1]

Stone[2] studied the carbohydrate constituents of the sweet potato and proved the presence of saccharose among the sugars. Other previous investigations have been concerned only with the general composition. Consequently we selected the sweet potato as the object of our second study on this subject, and investigated the nature of the sugars.

Preparation of the syrup:

100 gm. of the finely pulverized material were extracted in a Soxhlet apparatus with ether. After evaporating the ether, the oil-free residue was placed in a 750 cc. Erlenmeyer flask, and extracted daily with 300 cc. of 95 per cent alcohol by heating in a boiling water bath, with a reflux condenser. About two weeks were required to remove the last traces of sugars. The combined extracts were filtered to remove the sediment which was formed on standing, and the filtrate was evaporated to a syrupy condition in a partial vacuum. The syrup was purified by shaking with absolute alcohol. The clear solution was decanted and evaporated down to about 10 cc.

The above method of preparing the syrup was once more repeated to get a sufficient quantity of the material for the investigation.

[1] The first paper, entitled On the Nature of the Sugars Found in the Tubers of Arrowhead, is published in this *Journal*, xv, p. 221, 1913.

[2] W. E. Stone: *Ber. d. deutsch. chem. Gesellsch.*, xxiii, p. 1406, 1890.

Qualitative tests of the syrup.

The syrup gave the following qualitative reactions:

1. It had a very sweet taste.

2. It reduced Fehling's solution strongly; after inversion with hydrochloric acid the reducing power is much enhanced, showing the presence of both reducing and non-reducing sugars.

3. It gave Molisch-Udransky reaction with α-naphthol and sulphuric acid.

4. It gave the characteristic blood-red color on heating with picric acid and caustic soda (reaction of Braun for glucose).

5. It gave the characteristic fiery red color of ketose with resorcin and hydrochloric acid (Selivanoff's reaction).

6. It gave Pinoff's reaction with ammonium molybdate and acetic acid.

7. It did not show any pentose reaction by the phloroglucin method.

8. No mucic acid was produced upon oxidation with nitric acid.

9. Saccharic acid was detected as acid potassium salt in the oxidized solution of the syrup.

10. It rotated the plane of polarization toward the right; after inversion it was slightly levorotatory.

11. It produced no characteristic mannose phenylhydrazone with phenylhydrazine, either in the original or in the inverted syrup. When the mixture in both cases was warmed in a boiling water bath with acetic acid, the yellowish, well crystallized osazone was produced.

12. Two drop portions of the syrup were placed on object glasses, and inoculated respectively with crystals of glucose, fructose, maltose, and sucrose. After twenty-four hours the solution which had been inoculated with sucrose showed the formation of new crystals, while the others remained unchanged.

From the above tests it is evident that the syrup contains both reducing and non-reducing sugars, and that the presence of fructose and sucrose is highly probable. Moreover, it is safe to conclude that the presence of pentose and mannose molecules is excluded, since no characteristic reactions could be found, as above mentioned.

Isolation of sucrose.

When the syrup was left untouched for about twenty-four hours, fine crystals were abundantly formed in it. A small amount of 95 per cent alcohol was then added to the syrup, the mixture was filtered and the precipitate washed with absolute alcohol and ether. The sugar thus obtained was recrystallized from alcohol. After drying in a vacuum over sulphuric acid the purified sugar was about 2 gm. in weight.

0.6439 gm. of the sugar was dissolved in water, made up to 25 cc., and polarized in a 200 mm. tube, in a Schmidt and Haensch half shadow polariscope. A dextrorotation of 9.9 on the scale was observed. The specific rotary power is

$$[\alpha]_D = \frac{9.9 \times 0.346 \times 25}{0.6439 \times 2} = +66.5 \text{ (at } 20°).$$

The specific rotary power indicates that the sugar under examination is sucrose.

Phenylosazone tests.

The mother liquor filtered from the sucrose crystals was concentrated again into a syrup, but it did not show any sign of forming new crystals, even after one week's standing. An attempt was then made to separate and detect the sugars as phenylosazones.

1 gm. of the syrup, 2 gm. of phenylhydrazine hydrochloride, 3 gm. of sodium acetate, and 20 cc. of water were mixed and heated in a boiling water bath. After fifteen minutes yellowish crystals had been produced. At the end of one hour and a half the heat was removed and the crystals were examined under the microscope. None of the other forms, besides the stellate form of yellow needle-shaped crystals which coincides with that of phenylglucosazone was observed. When cooled it was filtered and washed with a little water. Upon recrystallization from 60 per cent alcohol and drying over sulphuric acid in a vacuum, the amount was 0.85 gm. The melting point was determined and found to be 204°, which coincides with that of phenylglucosazone. Consequently, the osazone under examination is phenylglucosazone.

1 gm. of the syrup was dissolved in 20 cc. of water and inverted with hydrochloric acid in a boiling water bath for about thirty minutes. After it was neutralized with sodium carbonate, 2 gm. of phenylhydrazine hydrochloride and 3 gm. of sodium acetate were added and the mixture was heated in a boiling water bath, exactly in the same manner as above described. After heating for one hour and a half, the crystals were examined under the microscope, but they were all uniform and quite identical

with those of phenylglucosazone which was obtained in the previous experiment. When cooled, it was filtered and washed with a little water. The yellow crystals thus obtained were recrystallized from dilute alcohol and dried over sulphuric acid in a vacuum. The product weighed 1.06 gm., and the melting point was found to be 204°. The crystalline form and melting point indicated that the osazone obtained was phenylglucosazone without admixture of other osazones.

The osazone test which was made to separate and detect the sugars in the syrup thus did not yield results differing from those obtained by the qualitative tests, as already described. But, as a result of this experiment, the presence of maltose is excluded, since maltose if present would have formed an osazone of a melting point very similar to that of glucosazone, but easily distinguishable from the latter by its crystalline form.

SUMMARY.

1. Sugar of the sweet potato tubers is made up of both reducing and non-reducing sugar.

2. The reducing sugar consists of both glucose and fructose, while the non-reducing sugar is sucrose.

3. The presence of pentose, galactose, and mannose molecules is excluded. The presence of maltose is also excluded.

ON THE NUCLEIN BASES FOUND IN THE SHOOTS OF ARALIA CORDATA.

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The present paper is a statement of our study on the chemical nature of the nuclein bases found in the shoots of *Aralia cordata*, or Udō, which are widely consumed in Japan as an article of food.

A search through the literature concerning *Aralia cordata* failed to yield any results of special investigations on the nitrogenous constituents of the shoots, beyond a brief article by Takeuchi[1] on their general composition and carbohydrate constituents. His results are as follows:

	FRESH SUBSTANCE	DRY MATTER
	per cent	*per cent*
Water	94.50	.....
Crude protein	1.10	19.97
Crude fiber	0.85	15.38
Ether extract	0.42	7.67
Ash	0.55	9.91
Total nitrogen	0.18	3.20
Protein nitrogen	0.11	2.01
Non-protein nitrogen	0.05	1.19
Protein	0.69	12.56
Dextrose	0.24	4.41
Sucrose	0.07	1.21
Starch	0.12	2.26
Pentosans	0.41	7.47
Galactans	0.03	0.53
Tannin	0.33	5.95

To determine the exact nature of the nuclein bases of the *Aralia* shoots, the following investigation was undertaken.

[1] T. Takeuchi: *Bulletins of the College of Agriculture*, Tokyo, vii, p. 465, 1906–08.

15 kg. of the fresh shoots were cut into small slices and thoroughly extracted with hot water, and the extract was treated with basic lead acetate. The filtrate from the lead compound was decomposed by hydrogen sulphide. The lead sulphide formed was filtered off, the filtrate was then neutralized with a little ammonia, and was slowly evaporated over a water bath, a little ammonia being added from time to time during evaporation to maintain the neutrality of the evaporating fluid. When the whole filtrate was concentrated to about a liter, sulphuric acid was added until its content reached 5 per cent. To this acidified fluid phosphotungstic acid was added, and the mixture well shaken. After twenty-four hours the precipitate was filtered off, thoroughly washed with 5 per cent sulphuric acid, and decomposed by baryta, in the usual manner. The barium phosphotungstate formed was filtered off, and the filtrate, freed from baryta by carbon dioxide, was again evaporated to a small volume. After neutralization with nitric acid, concentrated silver nitrate solution was added, and the precipitate formed was collected on a filter. The precipitate was then transferred to a beaker, an excess of ammonia was added, and it was left to stand for twenty-four hours. The silver compound formed was decomposed with dilute hydrochloric acid, and filtered. When the filtrate was evaporated to a small volume, an excess of ammonia was added, and it was left untouched for twenty-four hours. The yellowish powder formed was separated, washed, then dissolved in a small quantity of water, and a slight excess of saturated picric acid solution was added. After twenty-four hours' standing, beautiful yellow crystals were produced. After drying over sulphuric acid in a vacuum, the product weighed about 0.1 gm., and its decomposition point was found to be 189°. For the analysis we again dried the crystals in a vacuum at 100° and obtained the following result:

0.0623 gm. of substance gave 0.01824 gm. N.

		N *per cent*
$C_5H_5N_5O,C_6H_2(NO_2)_3OH$	Calculated:	29.47
	Found:	29.28

From this result we may conclude that the crystals were guanine picrate.

The filtrate from guanine was acidified with hydrochloric acid, and evaporated to dryness. The residue was then treated with alcohol to remove the hydrochloric acid as much as possible, and dissolved in water. When the ammoniacal solution of silver nitrate was added to the solution, a white precipitate of silver compounds was produced. The precipitate was collected on a filter and transferred to a beaker. After being decomposed with hydrochloric acid, the solution was freed from silver chloride by filtration and then evaporated to a syrup. The syrup was again treated with alcohol to remove the hydrochloric acid, water was added to the syrup, and it was left to stand for twenty-four hours at 40°.

The yellowish powder produced was collected on the filter, washed with water, and dried in a vacuum over sulphuric acid. The amount of powder obtained was too small to carry out an elementary analysis, but it gave the well known Weidel's and Strecker's xanthine reactions. Consequently there is no doubt that the powder obtained was xanthine.

The filtrate from the xanthine was left untouched for nearly forty-eight hours in a cool place, but it did not show any signs of forming crystals. The concentrated picric acid solution was then added to this filtrate, and the mixture was left untouched again for about twenty-four hours. Still it did not produce even one crystal of picrate. Therefore it is clear that the filtrate does not contain either adenine or hypoxanthine.

SUMMARY.

We have tried to separate the nuclein bases of *Aralia cordata*, and proved the presence of guanine and xanthine. Adenine and hypoxanthine were not detected.

A CONTRIBUTION TO THE STUDY OF THE AMINO-ACID CONTENT OF THE BLOOD.

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INTRODUCTION.

In spite of the numerous contributions on this subject, the question of the form in which alimentary nitrogen is absorbed by the intestine still remains disputed.

Some authors (Abderhalden and his collaborators[1] and one of us[2]) are inclined to regard as indispensable the breaking up of foreign albuminous molecules, if not into their final fragments, at least into fragments sufficiently simple to permit the organism to reconstruct its own proteins at their expense. Other physiologists, on the contrary, (von Bunge,[3] Nolf[4]) hold rather to the idea that disintegration is not carried far in the intestine.

Abderhalden and London[5] have brought to light the important fact that digestive processes permit, by virtue of the successive and combined action of the proteoclastic enzymes of the various secretions in the gastro-intestinal canal, division of proteins almost to their final constituents. In addition Abderhalden,[6] continuing the experiments of Otto Loewi[7] and of Henriques and Hansen,[8] has shown, in collaboration with Rona, Oppler, and

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[1] E. Abderhalden: *Ztschr. f. physiol. Chem.*, xliv, pp. 17–52, 1905; *Lehrbuch der physiologischen Chemie*, 3d edition, pt. i, Berlin, 1914.

[2] E. Zunz: *Rev. de l'Univ. de Bruxelles*, viii, pp. 755–770, 1903.

[3] G. v. Bunge: *Lehrbuch der Physiologie des Menschen*, Leipsic, 1901, ii, p. 205.

[4] P. Nolf: *Jour. de physiol. et de path. gén.*, ix, pp. 925–938 and 957–968, 1907.

[5] Abderhalden: *loc. cit.* E. S. London: *Physiologische und pathologische Chymologie*, Leipsic, 1913.

[6] Abderhalden: *loc. cit.* J. Effront: *Les catalyseurs biochimiques dans la vie et dans l'industrie*, Paris, 1914.

[7] O. Loewi: *Arch. f. exper. Path. u. Pharmakol.*, xlviii, pp. 303–330, 1902.

[8] V. Henriques and C. Hansen: *Ztschr. f. physiol. Chem.*, xliii, pp. 417–446, 1904–05.

other workers,[9] that it is easily possible to nourish dogs by means of the advanced products of the disintegration of albuminous material.

On the other hand, Nolf and Honoré,[10] as well as one of us,[11] have observed, that with an equal nitrogen content, the average coefficient of nitrogen absorption in the small intestine, isolated *in situ* in the dog, is less for solutions of substances free from biuret than for those of proteoses. However, if one takes account of the transformations undergone by the splitting products of the proteins in the digestive tube, and of the numerous factors which intervene in the phenomena of absorption, these experiments do not conclusively demonstrate a more rapid intestinal absorption of proteoses than of substances free from biuret. Several months ago Messerli[12] compared, in a dog with a Thiry-Vella fistula, the rapidity of intestinal absorption of several solutions. This author did not discover any appreciable difference in this respect between the first products of disintegration of albuminous substances (peptones) and ereptone (biuret-free); on the contrary, casein completely hydrolyzed was most slowly absorbed. Messerli concluded that it is by no means indispensable that proteins must be completely transformed in the intestines into their ultimate crystalline derivatives, and that a large part of the nitrogen of the food may be absorbed by the intestinal mucosa in the form of proteoses or peptones.

It is certain that in active digestion, the biuret-free substances predominate in the intestine.[13] This does not, however, teach us either the form in which the protein nitrogen of the food is absorbed, or the place in which the consecutive synthesis of the split products of the albuminous material occurs,—digestive canal itself, intestinal mucosa, circulating blood, tissues, or organs.

Within the past few years, most authors, basing their ideas on the work of Hofmeister,[14] Glaessner,[15] Kurajeff,[16] and Grossmann,[17] and on the inconclusive results of the study of the incoagulable biuret-free substances and other derivatives of the proteins in the circulating blood[18] located the

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[9] Numerous studies in *Ztschr. f. physiol. Chem.*, since 1904.

[10] P. Nolf and C. Honoré: *Arch. internat. de physiol.*, ii, pp. 85–115, 1904–05.

[11] Zunz: *Mém. de l'Acad. roy. de méd. de Belg.*, xx, pt. i, pp. 1–65, 1908.

[12] H. Messerli: *Biochem. Ztschr.*, liv, pp. 446–473, 1913.

[13] London: in *C. Oppenheimers Handb. d. Biochem.*, Supplement, Jena, 1913, pp. 404–432.

[14] F. Hofmeister: *Ztschr. f. physiol. Chem.*, vi, p. 69, 1882; *Biol. Centralbl.*, ii, p. 63, 1882–83.

[15] K. Glaessner: *Beitr. z. chem. Phys. u. Path.*, i, pp. 328–338, 1901.

[16] D. Kurajeff: *ibid.*, iv, pp. 476–485, 1904.

[17] J. Grossmann: *ibid.*, vi, pp. 192–205, 1905.

[18] The history of this question is well given in the work of H. Hohlweg and H. Meyer: *Beitr. z. chem. Phys. u. Path.*, xi, pp. 381–403, 1908.

synthesis of proteins in the gastro-intestinal mucosa. Abderhalden has ventured the opinion that the amino-acids, absorbed by the gastro-intestinal mucosa, were either directly synthesized in the intestinal wall to the specific proteins of the organism, or utilized immediately in other ways. But in support of this theory the eminent physiologist of Halle brings forth only presumptions, supported by the non-appearance of amino-acids in the blood during digestion, a negative finding, due, as is now shown, to the insufficiency of the methods in use at that time. All positive proof is lacking. Abderhalden and London[19] have not shown an increase in albumin in the intestinal wall during digestion. Rona[20] did not observe any formation of proteins from amino-acids in surviving intestine. Folin and Denis[21] have not found an augmentation in the ammonia content of the portal vein indicating an immediate deamination of the proteins of the food.

Nevertheless, previous to these later experiments, the presence of amino-acids in the blood under normal[22] or experimental conditions[23] had been shown. Bingel[24] isolated glycocoll from ox blood, probably originating not from intestinal absorption, but from cellular metabolism. Abderhalden[25] has, as a matter of fact, finally demonstrated the presence of glycocoll, arginine, lysine, histidine, proline, leucine, valine, aspartic acid, and glutamic acid in the normal blood of the ox or horse. By means of the method of vital dialysis, Abel[26] has accomplished the same result with the dog.

We now come to the question concerning the influence which digestion exerts on the level of the amino-nitrogen content of the blood. The increase of residual nitrogen shown under these circumstances[27] renders probable an increase in amino-nitrogen during the digestive process. Furthermore, Hohlweg and Meyer have studied the nitrogen not precipitated by tannin, nitrogen which corresponds closely to the nitrogen of amino-acids. These authors found, after a fast of seven days, 1 to 9 mg. (average 6.4) of nitrogen non-precipitable by tannin per 100 cc. of dog blood. This value reached 7 to 20 mg. (average 13 mg.) from two to seven hours

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[19] Abderhalden and London: *Ztschr. f. physiol. Chem.*, lxv, pp. 251–255, 1910.

[20] P. Rona: *Biochem. Ztschr.*, xlvi, pp. 307–316, 1912.

[21] O. Folin and W. Denis: this *Journal*, xi, pp. 161–167, 1912.

[22] W. H. Howell: *Am. Jour. Physiol.*, xvii, pp. 273–279, 1906–07.

[23] E. Abderhalden, A. Gigon, and E. S. London: *Ztschr. f. physiol. Chem.*, liii, pp. 113–118, 1907.

[24] A. Bingel: *ibid.*, lvii, pp. 382–388, 1908.

[25] Abderhalden: *ibid.*, lxxxviii, pp. 478–483, 1913.

[26] J. J. Abel, L. G. Rowntree, and B. B. Turner: *Jour. Pharmacol. and Exper. Therap.*, v, pp. 275–316, 1913–14.

[27] G. v. Bergmann and L. Langstein: *Beitr. z. chem. Phys. u. Path.*, vi, p. 27, 1904. E. P. Cathcart and J. B. Leathes: *Jour. Physiol.*, xxxiii, pp. 462–475, 1905–06. H. Pringle and W. Cramer: *ibid.*, xxxvii, pp. 158–164, 1908. Hohlweg and Meyer: *loc. cit.*

after ingestion of horse meat. This variety of nitrogen is present then, during digestion, in quantities about double those seen in the same animal during fasting. The values for nitrogen not precipitated by tannin observed during fasting and during digestion correspond very closely, as will be shown, to those obtained under the same conditions by the modern methods for determination for amino-acids, all being naturally a trifle higher. The ingenious method of Folin and Denis[28] for determination of residual nitrogen has permitted these authors to show an increase in this form of nitrogen, bearing chiefly on the amino-acids, either during normal digestion or after injection of amino-acids into the stomach or intestines, ligated at the two ends.[29]

The work to which we have alluded furnishes us with only indirect presumptive evidence of an increase in the amino-nitrogen content of the blood during digestion. The interesting researches of Abderhalden and Lampé[30] have already brought a more direct proof. According to these authors, the substances reacting with ninhydrin (and they are primarily amino-acids) increase markedly in the blood during digestion, and may not be, under certain experimental conditions, found at other times.

Delaunay[31] was the first to determine the amino-nitrogen of the blood in an accurate quantitative manner. By means of the formol method of Sörensen, this author has established the following findings: (1) the blood and all tissues and organs contain in all of a series of animals appreciable quantities of amino-nitrogen; (2) during digestion of a meal rich in proteins the amino-acid content of the blood increases markedly; (3) this increase is more marked in the portal vein than in arterial blood, and especially more than in venous blood. Based on these experiments Delaunay has, since 1910, viewed an absorption of amino-acids by various tissues and organs as a regulatory mechanism of the amino-acid content of the blood, a mechanism in which the liver plays the principal part. Van Slyke and Meyer have, in addition, given experimental proof of the truth of the views brought forth by Delaunay. Abderhalden has recently defended ideas quite analogous to those of the distinguished savant of Bordeaux.

Van Slyke and Meyer[32] have used in their researches the gasometric method proposed by the first named. They have demonstrated the constancy of the amino-nitrogen content of the blood of the dog, which remains the same on several days of fasting. Either after ingestion of food, or after intravenous or intra-intestinal introduction of amino-acids the

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[28] Folin and Denis: *loc. cit.*, pp. 527–536.

[29] Folin and Denis: this *Journal*, xii, pp. 141–162, 1912. O. Folin and H. Lyman: *ibid.*, pp. 259–264, 1912.

[30] E. Abderhalden and A. E. Lampé: *Ztschr. f. physiol. Chem.*, lxxxi, pp. 473–507, 1912.

[31] H. Delaunay: *Thèse de Bordeaux,* 1910; *Compt. rend. Soc. de biol.*, lxxiv, pp. 767–769, 1913.

[32] D. D. Van Slyke and G. M. Meyer: this *Journal*, xii, pp. 399–410, 1912.

amino-nitrogen content of the blood is markedly increased. This increase is practically the same in arterial or venous peripheral blood—which is not in accord with the findings of Delaunay. The American authors have, however, as has Delaunay, observed during the digestive period an amino-nitrogen content greater in the portal vein and its ramifications than that of the peripheral arterial and venous system.

Costantino[33] has verified, by the method of Sörensen, the principal results of the important researches of Van Slyke and Meyer. Costantino has in addition studied the partition of amino-nitrogen between the plasma and corpuscles. During fasting the content of the corpuscles in amino-nitrogen corresponds to that of the plasma. During digestion the amino-nitrogen content of the corpuscles increases considerably, that of the plasma little or not at all. It may therefore be concluded that the cells are very permeable to amino-acids.[34]

There remain to be cited the communications of Dobrowolskaja[35] and of Wolkow.[36] These authors have employed the method of Sörensen. They have thus shown, in London's laboratory, the increase in amino-nitrogen content of the blood of the dog, especially in the portal circulation, during digestion of a meal rich in proteins. Dobrowolskaja has, furthermore, found an increase of the free amino-nitrogen in the urine of dogs receiving food to which glycocoll or other amino-acids are added.

In the numerous researches, the results of which we have summarized, but little attention has been paid to an important factor; *i.e.*, the influence of bleeding. Up to the present no one has used the gasometric method of Van Slyke to study the partition of amino-nitrogen between the corpuscles and plasma of the dog, either fasting or having received several hours previously a meal with a heavy or light protein content.

In view of the great interest presented by these questions, not only in regard to the gastro-intestinal absorption of the proteins, but also for the intimate cellular metabolism, it has appeared useful to us to introduce several experiments on this point.[37] We believe the gasometric method of Van Slyke to be preferable to the formol method of Sörensen.[38] We shall not enter here into a

[33] A. Costantino: *Biochem. Ztschr.*, li, pp. 91–96, 1913; lv, pp. 402–410, 1913.

[34] Costantino: *ibid.*, lv, pp. 411–419, 1913.

[35] N. A. Dobrowolskaja: *ibid.*, lvi, pp. 267–290, 1913; *Ztschr. f. physiol. Chem.*, lxxxvii, pp. 325–334, 1913.

[36] A. D. Wolkow: *ibid.*, lxxxvii, pp. 334–337, 1913.

[37] The results of these experiments have been summarized in a preliminary communication which appeared in the *Compt. rend. Soc. de Biol.*, lxxvi, pp. 437–439, 1914.

[38] Zunz: *Internat. Beitr. z. Path. u. Therap. d. Ernährungsst*, ii, pp. 372–412, 1911; v, pp. 1–25, 1913.

critical examination of the advantages and disadvantages or inconveniences of these two ingenious experimental methods. It will suffice to add that, following the final perfections added by Van Slyke to his procedure, the sources of error of that method are reduced in great measure, and one may attain, after having acquired the necessary familiarity with the various manipulations, results with truly remarkable concordance, without requiring, as previously, a large number of determinations on each sample.

TECHNIQUE.

We used dogs weighing from 7 to 10 kilos, after twenty-four hours' fasting, but, as is essential, having received water to drink freely. Usually the blood was taken from the carotid with a paraffined cannula. It was received in a vessel containing a proportion of 1 per cent sodium oxalate such that 100 cc. of total fluid contained 10 cc. of sodium oxalate solution. The oxalate content of the blood was thus one per thousand. The oxalate was prepared in 0.6 per cent salt solution, in order to prevent hemolysis by anisotonicity.

A part of each sample of blood was taken to determine, by means of the hematocrite of Kottmann, the relative volume of plasma and corpuscles.

The remainder was divided into two portions, one of which was submitted to prolonged centrifugalization (two hours at 2000 revolutions). There were then added, according to the method indicated by Van Slyke and Meyer,[39] nine volumes of 95 per cent alcohol to one volume of plasma of the centrifugalized portion or to one part of whole blood as withdrawn. 40 to 50 cc. of whole blood or of plasma were always used.

Thus two alcoholic solutions were obtained, the first representing the plasma, and the second the whole blood. The volume reached 400 to 500 cc. at the maximum. After standing twenty-four hours each of the mixtures was separated by filtration or centrifugalization into an alcoholic solution and precipitate. The solution was warmed in a water bath at a temperature not over 30 to 40°, until reduced to a small volume (about 5 cc.). To each of the residues was added a quantity of urease correspond-

[39] Van Slyke and Meyer: *loc. cit.*

ing to 1 cc. of the solution to 10 cc. of original blood, usually 4 to 5 cc., in addition to toluene.[40]

After twelve to fifteen hours' standing at room temperature, the residue, with the addition of urease and toluene, was treated by the method of Folin, in order to free it from ammonia, either preformed or from urea. Care was taken not to add kerosene or any other analogous liquid. Loss by bubbling was avoided by using very high wash bottles and passing through the current of air very gently. Under these conditions, to be sure of driving off all ammonia the current of air must be passed through for at least eight hours.

A known volume of each of the liquids treated by the method of Folin was taken, filtered, and the aliphatic amino-nitrogen content of the filtrate determined according to the gasometric method of Van Slyke, employing the micro-apparatus recently described by that author.[41]

We have not removed the lipoids from the alcoholic filtrates obtained from the whole blood or plasma. We have been able to convince ourselves that the presence of lipoids is not a source of error in the gasometric determination of amino-nitrogen, as Van Slyke has already shown.

We shall have occasion, in the course of the description of our experiments, to mention again several points in our technique.

EXPERIMENTS.

Preliminary considerations. To study the variations in the amino-nitrogen content of the blood during digestive processes one must be able to compare the values obtained under these circumstances with normal values. There are, however, not many contributions on this point. Therefore, in connection with

[40] The solution of urease was prepared according to the method of E. K. Marshall, Jr., (this *Journal*, xiv, pp. 283–290, 1913). 10 gm. of powdered Soya hispida were emulsified in 100 cc. of water. This was allowed to stand for several hours, taking care to shake it at several intervals. 10 cc. of $\frac{N}{10}$ HCl were added. A precipitate was thus formed, from which the supernatant fluid was obtained by immediate filtration. This filtrate constituted the solution of urease which served to transform the urea into ammonia. This solution added to toluene keeps well in the ice box for several days.

[41] Van Slyke: this *Journal*, xvi, pp. 121–124, 1913–14.

each experiment, the amino-acid content of the blood in the normal condition must be determined.

To this end we have proceeded in the following way: The dog was kept fasting for twenty-four hours, always being allowed to drink water freely. Blood was taken, and after three and one-half to four hours a second bleeding was done. After the first bleeding the animal received, per kilo of body weight, 25 grams either of uncooked beef, or of potatoes. Lean meat was used, freed as much as possible from fascia, tendons, and all non-muscular elements. With water a homogeneous purée of potatoes was prepared, which the dogs ate eagerly.

Each animal was bled at two intervals, a period of three and one-half to four hours intervening. A second factor must, in consequence, be considered,—the influence of the first bleeding. Wolkow and Delaunay claim to have found no such effect. Wolkow operated on dogs weighing 20 to 30 kilos; that is, two to six times the weight of the animals placed at our disposal. In addition, in order better to assure the accuracy of our results we have, as a rule, taken 100 to 125 cc. of blood from the carotid at the first bleeding (sometimes only 50 to 60 cc., exceptionally 165 cc.) from dogs of from 5.5 to 12 kilos. It was then indispensable to begin by assuring ourselves that under such circumstances the bleeding did not cause modifications in the content of amino-acid of the blood taken three and one-half to four hours later.

To facilitate the exposition of our results we shall divide our experiments into three groups concerning the influence (1) of bleeding, (2) of a meal of potatoes, (3) of a meal of meat, on the amino-nitrogen content of whole carotid blood, plasma, and corpuscles.

First series of experiments.

Influence of bleeding on the amino-nitrogen content of the blood.

Table I illustrates the results of five experiments carried out with the purpose of determining the influence of a preliminary bleeding on the amino-nitrogen content of carotid blood, plasma, and corpuscles. Between the two bleedings the dogs were allowed to drink water freely.

It is to be remarked that Dog D was unfortunately not under entirely normal conditions. The animal showed, after the second

TABLE I.

DOG	WEIGHT	FIRST BLEEDING				HRS. BETWEEN BLEEDINGS	SECOND BLEEDING				DIFFERENCE BETWEEN VALUES OBTAINED AT FIRST AND SECOND BLEEDINGS					
		Blood taken	Amino-nitrogen per 100 cc. of				Blood taken	Amino-nitrogen per 100 cc. of			Whole blood		Plasma		Corpuscles	
			Whole blood	Plasma	Cor-puscles			Whole blood	Plasma	Cor-puscles	Mg.	Per cent of first value	Mg.	Per cent of first value	Mg.	Per cent of first value
	kg.	*cc.*	*mg.*	*mg.*	*mg.*		*cc.*	*mg.*	*mg.*	*mg.*						
A	7.800	125	5.0	2.7	7.2	3½	125	6.7	2.9	10.4	+1.7	+34.00	+0.2	+7.41	+3.2	+44.44
B	11.250	125	4.0	1.8	7.3	3½	130	5.4	4.6	6.6	+1.4	+35.00	+2.8	+155.56	−0.7	−9.59
C	5.500	120	4.0	—	—	4	50	6.2	—	—	+2.2	+55.00	—	—	—	—
D	6.000	165	4.9	3.9	8.0	4	125	5.8	6.6	2.6	+0.9	+18.37	+2.7	+69.23	−5.4	−67.50
E	7.600	125	1.8	—	—	4	50	2.4	—	—	+0.6	+33.33	—	—	—	—

bleeding, a marked state of weakness, and was found dead the following morning. The blood contained only 24.5 per cent of corpuscles at the first bleeding and even less at the second, 19.5 per cent. The values found in this animal can be accepted only with certain reservations for the content of amino-nitrogen of the plasma and corpuscles at the first, and especially at the second bleeding.

One must discard Dog E as far as the absolute values for amino-nitrogen at the two consecutive bleedings are concerned, since the values obtained are much lower than those found not only in Dogs A and D, but also in the two other series. It seems to indicate, in that animal, a pathological state of hypoamino-acidemia.

Excluding this experiment, one is struck with the remarkable concordance presented by the results on the content not only of the whole blood, but also of the plasma and cells of dogs after twenty-four hours' fasting. The plasma shows a content of amino-nitrogen less than that of the whole blood, and especially than that of the corpuscles. We shall have occasion later to return to these points.

For the moment we shall concern ourselves only with the influence of bleeding. Table I shows in the most complete way that the removal of 120 cc. of blood from the carotid suffices to cause three and one-half to four hours later an increase of 18.37 to 55 per cent (average 33 per cent) in the amino-acid content of the blood. This fact is verified by Dog E in the state of hypoamino-acidemia.

The increase in amino-acids following a preliminary bleeding is manifested especially in the plasma.

The results are not concordant as far as the corpuscles are concerned. In Dog A the amino-nitrogen content of the corpuscles increases, though less in proportion than that of the plasma, after the first bleeding. On the contrary, in Dog B there is a slight diminution in the amino-nitrogen content of the corpuscles. In Dog D this diminution is markedly noticeable. It is true that in the last case we have an animal already anemic before the first bleeding. This bleeding, relatively large, since it corresponded to 2.65 per cent of the animal's weight, caused a state in which a fatal issue occurred at the end of several

hours after the second bleeding, following difficulty in blood regeneration.

In order to see whether the sudden removal, at the first bleeding, of a relatively large proportion of the blood volume did not enter, on account of the sudden fall in pressure thus brought about in the circulatory system, into the phenomena which manifest themselves by an increase in the amino-nitrogen content of the blood, we have in seven experiments injected into the jugular the same quantity of Ringer's solution, previously warmed to 37°, as the quantity of blood removed at the first bleeding. The intravenous injection was done at the same moment as the bleeding.

Table II demonstrates that the preceding hypothesis is justified in a certain measure, as it may be seen that four hours after the intravenous injection of Ringer's solution simultaneously with the first bleeding, in five out of seven experiments a diminution in the amino-nitrogen content of the blood occurs in place of the increase otherwise found. In one case the amino-acid content of the whole blood did not vary. In one case it increased slightly, in spite of the injection of Ringer's solution.

In the plasma the amino-nitrogen content diminished in one case, did not change in one other, and in the last two increased, just as when bleeding was done without concomitant intravenous injection of Ringer's solution. This increase is nevertheless less pronounced than in the latter cases.

Whether one injects Ringer's solution or not into the jugular during the first bleeding, the content of the corpuscles in amino-nitrogen is for the most part diminished four hours after that bleeding, but now and then undergoes an increase (Table II).

Second series of experiments.

Influence of a meal of potatoes on the amino-nitrogen content of the blood.

Table III shows a résumé of the results obtained in six experiments in which the dogs received purée of potatoes. The time after the meal at which the blood was taken from the carotid was always four hours. In Dogs Q and R bleeding before the meal was avoided, with the purpose of excluding the influence of hemorrhage on the digestive processes.

TABLE II.

DOG	WEIGHT	FIRST BLEEDING				SECOND BLEEDING				DIFFERENCE BETWEEN VALUES OBTAINED AT FIRST AND SECOND BLEEDINGS					
		Blood taken	Amino-nitrogen per 100 cc. of			Blood taken	Amino-nitrogen per 100 cc. of			Whole blood		Plasma		Corpuscles	
			Whole blood	Plasma	Corpuscles		Whole blood	Plasma	Corpuscles	Mg.	Per cent of first value	Mg.	Per cent of first value	Mg.	Per cent of first value
	kg.	*cc.*	*mg.*	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*	*mg.*						
F	7.500	125	4.6	—	—	50	3.6	—	—	−1.0	−21.74	—	—	—	—
G	10.200	125	4.6	—	—	50	3.5	—	—	−1.1	−23.91	—	—	—	—
H	11.600	130	4.0	2.7	6.1	130	4.6	2.9	7.7	+0.6	+15.00	+0.2	+7.41	+1.6	+25.21
I	10.400	130	4.3	—	—	50	4.3	—	—	0.0	—	—	—	—	—
J	11.800	130	5.0	1.8	10.9	130	4.6	1.9	9.8	−0.4	−8.00	+0.1	+5.56	−1.1	−10.10
K	8.300	130	5.1	3.5	8.3	130	4.8	3.1	8.1	−0.3	−5.89	−0.4	−11.42	−0.2	−2.41
L	6.350	130	4.8	4.0	6.6	130	4.4	4.0	5.3	−0.4	−8.34	0.0	0.00	−1.3	−19.70

TABLE III.

DOG	WEIGHT	FIRST BLEEDING				SECOND BLEEDING				DIFFERENCE BETWEEN VALUES OBTAINED AT FIRST AND SECOND BLEEDINGS					
		Blood taken	Amino-nitrogen per 100 cc. of			Blood taken	Amino-nitrogen per 100 cc. of			Whole blood		Plasma		Corpuscles	
			Whole blood	Plasma	Corpuscles		Whole blood	Plasma	Corpuscles	Mg.	Per cent of first value	Mg.	Per cent of first value	Mg.	Per cent of first value
	kg.	*cc.*	*mg.*	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*	*mg.*						
M	8.000	100	4.0	—	—	60	5.3	—	—	+1.3	+32.50	—	—	—	—
N	8.000	125	4.5	—	—	60	4.4	—	—	−0.1	− 2.22	—	—	—	—
O	8.100	100	4.6	—	—	60	6.4	—	—	+1.8	+39.13	—	—	—	—
P	11.250	125	4.5	2.8	6.7	125	5.1	4.9	5.4	+0.6	+13.33	+2.1	+75.00	−1.3	−19.40
Q	7.300					60	4.9	—	—						
R	9.200					125	4.0	2.6	6.6						

An increase in the amino-nitrogen content of the blood after ingestion of potatoes is seen only in the three dogs M, O, and P. However, these three animals had undergone a preliminary bleeding, and the absolute values and those relative to the increase in amino-nitrogen content of the blood are entirely analogous to those observed at the time of the second bleeding in Dogs A and D, which remained fasting during the three and one-half to four hours between the two bleedings.

In Dog P the increase in the amino-nitrogen content of the blood is only effected in the plasma, and the content of the corpuscles in amino-nitrogen is diminished, just as we have seen at the second bleeding in Dogs B and D, fasting between the two bleedings.

In the absence of any preliminary bleeding the amino-nitrogen content of the whole blood, of plasma, and of corpuscles of Dogs Q and R, having eaten of purée of potatoes, corresponds to the values found in fasting animals which have not undergone any other bleeding.

In Dog N, in spite of the preliminary bleeding and in spite of the meal, the carotid blood presents nearly the same amino-nitrogen content at the two bleedings.

From all the values given in Tables I and III and their comparison it may be clearly seen that the ingestion of purée of potatoes did not exert any influence on the amino-nitrogen content of the blood of the dogs examined.

Third series of experiments.

Influence of a meal of meat on the amino-nitrogen content of the blood.

It is entirely different in the case of ingestion of meat, as is shown by the figures in Table IV. Of six animals used in this series of experiments, three (Dogs S, T, and U) have undergone a preliminary bleeding immediately before the meal. This consisted of 25 grams of raw beef per kilo of animal. Four hours after the meal the blood was taken from the carotids of the six animals. In addition, in three of them (Dogs T, U, and V) 50 cc. of blood were taken from the jugular at the same time.

TABLE IV.

DOG	WEIGHT	FIRST BLEEDING				SECOND BLEEDING						DIFFERENCE BETWEEN VALUES OBTAINED AT FIRST AND SECOND BLEEDINGS					
		Blood taken	Amino-nitrogen per 100 cc. of			Carotid blood				Jugular blood amino-nitrogen		Whole blood		Plasma		Corpuscles	
			Whole blood	Plasma	Cor-puscles	Blood taken	Amino-nitrogen per 100 cc. of: Whole blood	Plasma	Cor-puscles	Mg. per 100 cc.	Per cent of content of carotid blood	Mg.	Per cent of first value	Mg.	Per cent of first value	Mg.	Per cent of first value
	kg.	*cc.*	*mg.*	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*	*mg.*								
S	12.000	125	5.3	2.6	8.0	125	11.0	4.0	18.9			+5.7	+107.55	+1.4	+53.85	+10.9	+136.2
T	11.100	130	5.8	3.3	10.4	130	12.4	8.1	21.2	6.7	54.03	+6.6	+113.85	+4.8	+145.4	+10.8	+103.9
U		50	4.3	—	—	125	12.4	5.4	20.6	8.5	68.50	+8.1	+188.37				
V						125	10.2	6.8	14.4	7.8	76.47						
W	8.500					50	10.1										
X						50	12.1										

Four hours after ingestion of meat the carotid blood shows a notable increase in its amino-nitrogen content, whether or not the animal was bled before the meal. This increase is far superior to that brought about by bleeding. This is an influence actually exerted by digestion of proteins on the amino-nitrogen content of carotid blood. It is well to call attention to the remarkable constancy presented by the amino-nitrogen content of carotid blood taken from dogs in active digestion of a meal rich in proteins. In place of values of 4 to 5 mg. per 100 cc. of blood found in fasting, there is seen four hours after ingestion of meat 10 to 12 mg. or two to three times as much.

On the other hand, the venous blood taken at the same time as the carotid in dogs during digestion of meat has an amino-nitrogen content (6.7 to 8.5 mg. per 100 cc.) intermediary between that of the carotid blood at the same time and during fasting. The content of venous blood represents, four hours after ingestion of meat, 54.03 to 76.47 per cent of the carotid blood at thesame moment.

These two facts (constancy of amino-nitrogen content of blood, and diminished content of venous blood in that variety of nitrogen) call attention to the probability of a regulatory mechanism excited during digestion in such a way as to prevent the presence of an excess of amino-acids in the circulating blood, while permitting the various tissues and organs to secure the final fragments of the albuminous molecules necessary to the rebuilding of their own proteins.

The increase in the amino-nitrogen content of the blood during digestion of a meal rich in proteins depends as much on the plasma as on the corpuscles. It occurs at times in the plasma alone (Dog T), at other times only in the corpuscles (Dog S).

If one recalls the differences presented by the two forms of food used, meat and potatoes, one should not be surprised by the findings in the animals from which blood was taken during digestion of one or the other meal. According to König,[42] potatoes contain on the average 1.99 per cent of their weight in the form of nitrogenous substances, against 20.5 per cent for lean

[42] J. König: *Die menschlichen Nahrungs- und Genussmittel,* 4th edition, Berlin, 1904, ii, p. 466 ff., p. 892 ff.

beef. Although the nitrogen of meat is almost entirely of protein nature, potatoes contain about 45 per cent of their nitrogen in the form of asparagin, amino-acids, and other products.

Partition of amino-nitrogen between plasma and corpuscles in various experimental conditions.

Based on the results given by Kottmann's hematocrite and on the values for amino-nitrogen found in plasma and in whole blood, one may calculate in an approximate way the partition of amino-nitrogen between plasma and corpuscles. Table V shows values thus calculated for all animals in which the plasma content of amino-nitrogen at both bleedings has been determined, with the exception of Dog D.

Comparison of results obtained under various experimental conditions.

It has appeared useful to us to summarize in Table VI the results from the dogs examined under various experimental conditions. We have not included in this table the figures for partition of amino-nitrogen between the corpuscles and plasma of Dog D, nor the figures of Dog E for total blood, since these animals were, as we have shown, under pathological conditions.

Table VII shows, in addition, the results tabulated in Table V, which are all relative to carotid blood.

In spite of several gaps, unfilled because of the insufficient number of animals examined, Tables VI and VII bring out clearly the following facts.

1. During fasting and in the normal state, blood taken from the carotid has a remarkable constancy in amino-nitrogen content. This content is from 4 to 5 mg. per 100 cc. This constancy is also seen for corpuscles and plasma separately considered. The amino-nitrogen content of the corpuscles exceeds that of the plasma.

2. A relatively copious bleeding is followed three and one-half to four hours later by an increase in the amino-nitrogen content of whole blood and of corpuscles. This increase corresponds on the average to 38.1 per cent of the original value for whole blood.

TABLE V.

Experimental manipulation during or immediately after first bleeding	Dog	Amino-nitrogen per 100 cc. of carotid blood at						Difference between values obtained at first and second bleedings					
		First bleeding			Second bleeding			Whole blood		Plasma		Corpuscles	
		Whole blood	Plasma	Corpuscles	Whole blood	Plasma	Corpuscles	Mg.	Per cent of first value	Mg.	Per cent of first value	Mg.	Per cent of first value
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*						
None	A	5.0	1.4	3.6	6.7	1.5	5.2	+1.7	+34.00	+0.1	+7.15	+1.6	+44.45
	B	4.0	1.1	2.9	5.4	2.7	2.7	+1.4	+35.00	+1.6	+145.45	−0.2	−7.41
Intravenous injection of Ringer's solution during first bleeding	H	4.0	1.6	2.4	4.6	1.8	3.0	+0.6	+15.00	+0.2	+13.33	+0.6	+25.00
	J	5.0	1.2	3.8	4.6	1.2	3.4	−0.4	−8.00	0.0	0.00	−0.4	−10.53
	K	5.1	2.3	2.8	4.8	2.1	2.7	−0.3	−5.89	−0.2	−8.70	−0.1	−3.57
	L	4.8	2.7	2.1	4.4	2.7	1.7	−0.4	−8.34	0.0	0.00	−0.4	−19.05
Ingestion of purée of potatoes immediately after first bleeding	P	4.5	1.6	2.9	5.1	2.9	2.2	+0.6	+13.33	+1.3	+81.25	−0.7	−24.14
Ingestion of meat immediately after first bleeding	S	5.3	1.3	4.0	11.0	2.1	8.9	+5.7	+107.55	+0.8	+61.54	+4.9	+122.50
	T	5.8	2.1	3.7	12.4	5.4	7.0	+6.6	+113.85	+3.3	+157.14	+3.3	+89.19

TABLE VI.

Condition of animal at time of removal of blood	Vessel from which blood was taken	Whole blood: No. of dogs examined	Whole blood: Amino-nitrogen per 100 cc. Minimum	Whole blood: Amino-nitrogen per 100 cc. Maximum	Whole blood: Amino-nitrogen per 100 cc. Average	Plasma: No. of dogs examined	Plasma: Amino-nitrogen per 100 cc. Minimum	Plasma: Amino-nitrogen per 100 cc. Maximum	Plasma: Amino-nitrogen per 100 cc. Average	Corpuscles: No. of dogs examined	Corpuscles: Amino-nitrogen per 100 cc. Minimum	Corpuscles: Amino-nitrogen per 100 cc. Maximum	Corpuscles: Amino-nitrogen per 100 cc. Average
			mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
After 24 hrs.' fasting	Carotid	18	4.0	5.8	4.6	10	1.8	4.0	2.8	10	6.1	10.9	7.95
$3\frac{1}{2}$ to 4 hrs. after bleeding	Carotid	4	5.4	6.7	6.25	2	2.9	4.6	3.75	2	6.6	10.4	8.5
4 hrs. after bleeding accompanied by intravenous injection of Ringer's solution	Carotid	7	3.5	4.8	4.25	4	1.9	4.0	2.97	4	5.3	9.8	7.7
4 hrs. after ingestion of purée of potatoes	Carotid	2	4.0	4.9	4.45	1			4.9	1			5.4
4 hrs. after bleeding followed by ingestion of purée of potatoes	Carotid	4	4.4	6.4	5.3	1			2.6	1			6.6
4 hrs. after ingestion of raw beef	Carotid	3	10.1	12.1	10.8	1			6.8	1			14.4
	Jugular	1			7.8								
4 hrs. after bleeding followed by ingestion of raw beef	Carotid	3	11.0	12.4	11.9	3	4.0	8.1	5.8	3	16.4	21.2	19.4
	Jugular	2	6.7	8.5	7.6								

TABLE VII.

CONDITION OF ANIMAL AT TIME OF REMOVAL OF BLOOD	NO. OF DOGS EXAMINED	AMINO-NITROGEN PER 100 CC. OF CAROTID BLOOD								
		Whole blood			Plasma			Corpuscles		
		Minimum	Maximum	Average	Minimum	Maximum	Average	Minimum	Maximum	Average
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
After 24 hrs.' fasting	9	4.0	5.8	4.8	1.1	2.7	1.7	2.1	4.0	3.1
$3\frac{1}{2}$ to 4 hrs. after bleeding	2	5.4	6.7	6.1	1.5	2.7	2.1	2.7	5.2	4.0
4 hrs. after bleeding accompanied by intravenous injection of Ringer's solution	4	4.4	4.8	4.6	1.2	2.7	1.9	1.7	3.4	2.7
4 hrs. after bleeding followed by ingestion of purée of potatoes	1			5.1			2.9			2.2
4 hrs. after bleeding followed by ingestion of raw beef	2	11.0	12.4	11.6	2.1	5.4	3.8	7.0	8.9	8.0

3. If an intravenous administration of Ringer's solution be given simultaneously with the bleeding, the effects of the latter on the amino-nitrogen content of the blood are prevented in the majority of cases, and a slight diminution in amino-nitrogen content may be seen.

4. Ingestion of purée of potatoes does not exert an appreciable influence on the amino-nitrogen content of the blood.

5. Ingestion of meat increases considerably the amino-nitrogen content of the carotid blood and to a lesser degree that of the jugular. Increase of amino-nitrogen content is greater in the corpuscles than in the plasma of the same carotid blood.

6. The increase in blood amino-nitrogen appears slightly greater in dogs which have been bled before the meat was eaten. At least the average amino-nitrogen content of whole blood, whether carotid or venous, taken four hours after meat ingestion, and that of the corpuscles of carotid blood, exceeds the values obtained in animals not bled before feeding.

7. The increase in amino-nitrogen content four hours after ingestion of meat occurs in both the plasma and corpuscles.

8. The amino-nitrogen content of blood taken from the carotid four hours after ingestion of meat presents a remarkable constancy. It varies between 10 and 12 mg. per 100 cc. of blood and represents more than double the amino-nitrogen content during fasting. The increase thus obtained corresponds on the average to 134.78 per cent of the original value for the dogs not bled before ingestion of meat, and 158.7 per cent for animals bled before the meal, or 147.83 per cent for all dogs which received meat to eat.

Of course nothing permits us to extend the preceding facts to other species of animals. Even for the dog they apply to the influence of bleeding and of the digestive processes only, in the state existing four hours after the meal. There is need for further investigations at various intervals either after bleeding or after a meal rich or poor in proteins.

It is necessary finally to remember that the estimation of amino-nitrogen was not made directly except on whole blood and plasma. The values indicated for corpuscles were calculated from these figures and on the determination of the relative volume of corpuscles and plasma by means of the hematocrite. Perhaps val-

ues determined directly on plasma and corpuscles would not be exactly the same.

Hirudin plasma of the dog, taken during fasting, has an amino-nitrogen content analogous to that for oxalated plasma. In three experiments 100 cc. of hirudin plasma contained respectively 1.4, 2.0, and 2.1 mg. of amino-nitrogen.

GENERAL CONSIDERATIONS.

Many facts of interest appear to us to result from the researches here described. The amino-nitrogen of arterial blood is practically constant in dogs kept fasting for twenty-four hours. In our experiment it was 4 to 5 mg. per 100 cc. of blood, with the exception of Dog E. In this case it was in all probability a pathological condition. Under the same experimental conditions and by the same method of study, Van Slyke and Meyer have found 3.2 to 5.4 mg. of amino-nitrogen per 100 cc. of arterial blood. The results of the experiments of Costantino with the formol method of Sörensen agree closely with those of Van Slyke and Meyer and with ours.

The organism tends to maintain the concentration of blood and the constancy of the composition of this medium. The regulatory mechanism of the composition of the blood has been the object of many studies, among which it will suffice to mention those of Leathes,[43] Achard and Loeper,[44] Asher,[45] and Terroine.[46]

The amino-nitrogen of the blood tends, in the normal dog, to remain at a constant level except during periods of digestion. Delaunay[47] first showed that various tissues contain determinable quantities of amino-nitrogen. The important work of Van Slyke and Meyer[48] confirms this point. They showed, furthermore, that the tissues take from the blood amino-acids when the latter

[43] J. B. Leathes: *Jour. Physiol.*, xix, pp. 1–14, 1895–96.

[44] C. Achard and M. Loeper: *Compt. rend. Soc. de biol.*, liv, pp. 337–338, 1902. Loeper: Mécanisme régulateur de la composition du sang, *Thèse de Paris*, 1903.

[45] L. Asher: *Der physiologische Stoffaustausch zwischen Blut und Geweben*, Jena, 1909.

[46] E. F. Terroine: *Compt. rend. Soc. de biol.*, lxxvi, pp. 523–526, 1914.

[47] Delaunay: *loc. cit.*

[48] Van Slyke and Meyer: this *Journal*, xvi, pp. 197–212, 1913–14.

exceed the normal concentration. The muscles and various organs appeared to possess a maximum capacity for absorption of amino-acids, less marked in the muscles than in the liver, kidneys, and intestines. The liver was shown to destroy rapidly the excess of amino-acids, probably by the process of deamination, with the formation of urea.[49] Perhaps the liver also intervenes in another way. In any case, as Münzer and Winterberg[50] had already maintained in 1894, the liver seems to be the principal location of catabolism of the derivatives of proteins not utilized in tissue formation.

Comparing the amino-nitrogen content of the muscles, liver, kidneys, spleen, and intestines of dogs sacrificed five hours to twelve days after the last meal, Van Slyke and Meyer found either no appreciable difference in the values or a tendency to their increase during fasting.[51] One is thus forced to admit, with these authors, that the amino-acids constitute an intermediary phase not only in the synthesis of proteins of each tissue, but also in their breaking down. During fasting the amino-acids of the blood come without doubt from the internal metabolism of the cells of the various tissues and organs of the body.

The constancy of the amino-acid content of the blood in the same species of animal is evident also from the experiments of Rosenberg[52] on the ox and on man. It seems, according to the researches of that author and also according to those of Delaunay,[53] that the amino-acid content of the blood varies in different species of animals.

In the dog the plasma and corpuscles present also a remarkable constancy in amino-nitrogen values. The plasma holds 2 to 3 mg. of this nitrogen per 100 cc., the corpuscles 7 to 8 mg. Costantino has in addition already shown that the corpuscles

[49] Van Slyke and Meyer: *ibid.*, xvi, pp. 213–229, 1913–14. This clearly explains certain results obtained by Abderhalden, Gigon, and London (*loc. cit.*).

[50] E. Münzer and H. Winterberg: *Arch. f. exper. Path. u. Pharmakol.*, xxxiii, pp. 164–197, 1893–94.

[51] G. Buglia and A. Costantino: *Ztschr. f. physiol. Chem.*, lxxxiv, pp. 243–253, 1913. Van Slyke and Meyer: this *Journal*, xvi, pp. 231–233, 1913–14.

[52] A. H. Rosenberg: *Biochem. Ztschr.*, lxii, pp. 157–160, 1914.

[53] Delaunay: *loc. cit.; Compt. rend. Soc. de biol.*, lxxiv, pp. 641–642, 1913.

have an amino-nitrogen content higher than that of the plasma. In the fasting dog Costantino and Delaunay have obtained by Sörensen's method values slightly higher than ours for the amino-nitrogen content of plasma, 4 to 5 mg. per 100 cc. This seems to depend on the difference between the two analytical methods used, as one always obtains, under the same conditions, higher values for amino-nitrogen by the method of Sörensen than by that of Van Slyke.[54] An indirect confirmation of the greater content of the corpuscles in amino-nitrogen is found in the communications of various authors who have studied either the residual nitrogen of the blood or the labile and free amino-nitrogen of the blood or plasma not freed previously of its proteins.[55]

Various recent contributions, which we can not here discuss in detail, seem to show that the amino-acids intervene not only in the intracellular metabolism of proteins, but also in that of the carbohydrates, and possibly in that of the fats. Lusk[56] has attributed the increase in metabolism after ingestion of meat to the stimulant action exerted by catabolic products of the amino-acids on cellular protoplasm. This author believed that the excitant action of these products plays a considerable part in the metabolism of the dog at rest or sleeping. He has in a recent article[57] abandoned this view. He believes now that the stimulant is some other product than amino-acids, since the latter do not markedly increase in the tissues during digestion.

It is very important to establish in a precise way the normal amino-nitrogen content of human blood. It is probable that under certain conditions a hyper- or hypoaminoacidemia may exist. We have given an example of hypoaminoacidemia in Dog E (Table I). Gorchkoff, Grigorieff, and Koutoursky[58] have established the occurrence of hyperaminoacidemia in man during atrophic cirrhosis of

[54] E. Abderhalden and F. Kramm: *Ztschr. f. physiol. Chem.*, lxxvii, pp. 425–434, 1912. Zunz: *Bull. de l'Acad. roy. de méd. de Belg.*, series 4, xxvi, pp. 282–318, 1912.

[55] P. Brodin: *Compt. rend. Soc. de biol.*, lxxvi, pp. 289–291, 1914. C. Achard and E. Feuillié: *ibid.*, lxxvi, pp. 253–254, 1914. H. Bierry, R. Hazard, and A. Ranc: *ibid.*, lxxvi, pp. 261–262, 1914.

[56] G. Lusk: this *Journal*, xiii, pp. 155–183, 1912–13.

[57] M. B. Wishart: this *Journal*, xx, p. 538, 1915.

[58] M. Gorchkoff, W. Grigorieff, and A. Koutoursky: *Compt. rend. Soc. de Biol.*, lxxvi, pp. 454–457, 1914.

the liver with icterus and in posthemorrhagic anemia. In the first case the liver does not carry on suitably its deaminizing function and does not transform sufficiently well into other forms the amino-acids in the circulation. As to the hyperaminoacidemia of the posthemorrhagic condition, it belongs probably to the process of regeneration.

We have established this result of hemorrhage in all of a series of animals four hours after the first bleeding. In general, it does not produce any increase in the amino-nitrogen content of the blood and on the contrary usually a diminution of one immediately follows the first bleeding with an abundant intravenous injection of Ringer's solution. The increase in the amino-nitrogen content of the blood, after bleeding four hours previously, therefore depends in great part on the sudden lowering of pressure thus produced. Regenerative processes, prevented by the concomitant injection of Ringer's solution, also doubtless intervene.

It has been known for a long time that a copious hemorrhage brings about certain modifications in the composition of blood. According to Claude Bernard,[59] von Mering,[60] Schenk,[61] and Rose,[62] hyperglycemia is observed under those conditions. Mauriac[63] has shown a hypercholesterinemia after large hemorrhages.

Delaunay and Wolkow have not found any modification in the amino-nitrogen content of the blood after bleeding. The differences between their results and ours are due without doubt to the proportion of blood taken suddenly from the organism. It must reach a considerable amount to produce a hyperaminoacidemia.[64]

[59] C. Bernard: *Leçons sur le diabète*, Paris, 1877.

[60] von Mering: *Arch. f. Physiol.*, pp. 379–415, 1877.

[61] F. Schenk: *Arch. f. d. ges. Physiol.*, lvii, pp. 553–572, 1894.

[62] U. Rose: *Arch. f. exper. Path. u. Pharmakol.*, l, pp. 15–46, 1903. B. Oppler and P. Rona: *Biochem. Ztschr.*, xiii, pp. 121–131, 1908.

[63] P. Mauriac: *Compt. rend. Soc. de biol.*, lxxiii, pp. 675–677, 1912.

[64] The interesting experiments of Dobrowolskaja, who carried out repeated bleedings in the dog under various conditions, should be mentioned here. He has studied the effect of the removal of large quantities of blood on the digestive processes, but as he operated under special conditions his animals are not, therefore, comparable with ours. (Dobrowolskaja: *loc. cit.; Biochem. Ztschr.*, xxxiii, pp. 73–152, 1911.)

Viola and Jona[65] have shown a considerable diminution in the alkalinity of the blood after bleeding. These authors attribute this to the invasion of the blood by acid products of metabolism of tissues. According to Fuchs[66] the metabolism is increased after copious bleeding. This author[67] in addition calls attention to the increase, under these circumstances, in the elimination of amino-acids in the urine, titrated by the method of Sörensen. This hyperaminoaciduria seems to indicate the same changes as hyperaminoacidemia, following an abundant bleeding or hemorrhage.

After bleeding the plasma often presents an increase in amino-acid content greater than that of the corpuscles. These may even show a diminution in amino-nitrogen content. Perhaps the inconstancy of these results is due to modification of the permeability of red cells and leucocytes, brought about by bleeding.

We now come to the phenomena that occur during digestive processes. Our experiments fully confirm the notable increase in amino-nitrogen content of blood after a meal rich in proteins, already shown by several writers. Without wishing to enter here into the problem of the absorption of albuminous substances, we may recall that only lately Abderhalden[68] admits a direct passage, more or less partial, of amino-acids into the blood.

The few studies which we have made to compare the amino-nitrogen content of arterial blood with that of venous blood demonstrate the retention of amino-acids by the tissues during digestion. Thus is explained why arterial blood during digestion has an amino-nitrogen content greater than venous blood taken at the same time. Delaunay has shown the same fact. On the contrary, Costantino and Van Slyke and Meyer have observed nearly equal values for amino-acid in the entire peripheral circulation, even in animals in the midst of the period of digestion. It is impossible for the moment to explain this divergence between the results of various authors who have studied this point.

All those who have studied the amino-nitrogen content of the blood taken from the portal vein during digestion are in accord

[65] G. Viola and G. Jona: *Arch. de physiol.*, xxvii, pp. 37–44, 1895.

[66] D. Fuchs: *Arch. f. d. ges. Physiol.*, cxxx, p. 156, 1909.

[67] Fuchs: *Ztschr. f. physiol. Chem.*, lxix, pp. 483–490, 1910.

[68] Abderhalden: *Lehrbuch der physiologischen Chemie*, 3d edition, pt. i, Berlin, 1914.

in finding that the values for amino-nitrogen are greatly increased, and that they may reach double the content of this variety of nitrogen in the blood of the vena cava or carotid, already increased in comparison with normal.

The differences presented by the amino-nitrogen content of the blood in the arterial system, the peripheral venous system, and the portal vein in the dog during active digestion are supported by the findings of Van Slyke and Meyer following intra-arterial or intravenous injection of amino-acids. They demonstrate, on the one hand, that the various tissues remove a part of the amino-acids circulating in the blood, and, on the other hand, that the liver retains and transforms these substances.

As Costantino has already shown, the increase in the amino-nitrogen content after a meal rich in proteins is especially marked in the corpuscles. Nevertheless, contrary to the opinion of that author, the plasma also takes an appreciable part, its amino-nitrogen content remaining always less than that of the corpuscles. The permeability of the blood corpuscles, still so little understood, certainly intervenes in the explanation of these phenomena.

SUMMARY.

1. In the dog the amino-nitrogen content of whole blood, of plasma, and of corpuscles shows under normal conditions a remarkable constancy.

2. Copious bleeding brings on an increase in the amino-nitrogen content of the blood. If, at the same time as the bleeding, an intravenous injection of Ringer's solution is given, the amino-nitrogen content of the blood tends, on the contrary, in the majority of cases to diminish.

3. The amino-nitrogen content of the blood does not change during the process of digestion of a meal poor in proteins (purée of potatoes).

4. During the digestion of a meal rich in proteins (raw beef), we observe, in agreement with the authors mentioned above, a very marked increase in the amino-acid content of the blood. This increase is more marked in the corpuscles than in the plasma.

5. During digestion of a meal rich in proteins, the content of venous blood in amino-nitrogen is lower than that of arterial blood.

SOME NEW CONSTITUENTS OF MILK.[1]

FIRST PAPER.

THE PHOSPHATIDES OF MILK.

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(Received for publication, May 11, 1915.)

In making the large quantities of protein-free milk required for the feeding experiments in progress in this laboratory, opportunity has been afforded to make several observations on some of the constituents of milk which are present in very small proportion. The fact that milk contains all of the nutrients essential for growth, and the increasing recognition of the fact that other still unknown substances than proteins, fats, carbohydrates, and inorganic salts containing iron, calcium, magnesium, potassium, sodium, phosphorus, sulphur, and chlorine, are necessary for normal growth, makes a knowledge of every constituent of milk, present in however small amount, a matter of especial interest and possible importance. We propose, therefore, to record in this series of papers some facts which we have already ascertained and hope to add others in the future, as opportunity presents.

It has frequently been stated that milk contains phosphatides.[2] On the other hand, Schlossmann[3] concluded that the phosphorus

[1] The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[2] Compare N. A. Tolmatscheff: *Med. chem. Untersuch.*, ii, p. 272, 1867. J. Stoklasa: *Ztschr. f. physiol. Chem.*, xxiii, p. 343, 1897. W. Koch and H. S. Woods: this *Journal*, i, p. 203, 1905–06. W. Koch: *Ztschr. f. physiol. Chem.*, xlvii, p. 327, 1906. R. Burow: *ibid.*, xxx, p. 495, 1900.

[3] A. Schlossmann: *Arch. f. Kinderh.*, xl, p. 18, 1904.

extracted by boiling alcohol arose from the casein and was not to be accepted as evidence of the presence of phosphatides in milk. Recently Njegovan[4] has reached the same conclusion. The question of the occurrence of phosphatides in milk has again been made the subject of study by Brodrick-Pittard,[5] who found that even when every precaution has been taken to exclude the presence of water during the extraction, an amount of phosphorus is dissolved by ether equal to 0.0180 gram of lecithin per 100 cc. of milk, or about 0.14 per cent of the solids. By subsequent extraction with chloroform phosphorus equal to 0.013 gram of lecithin was obtained, equal to 0.10 per cent more of the solids. If, however, the chloroform extraction preceded that with ether all of this phosphorus was removed by the chloroform and none by the ether. From these facts Brodrick-Pittard concludes that "at least two lecithins occur in milk which are distinguished by a different solubility in the solvents employed."

In all of these investigations no chemical evidence of the nature of the substances containing the ether-soluble phosphorus has been offered, so that the presence of phosphatides is based wholly on the assumption that such phosphorus belongs to compounds similar to, or identical with, the phosphatides obtained from other sources, notably egg-yolk and brain. We thus have no direct evidence that milk contains any substance belonging to the group of phosphatides.

From the failure to extract phosphorus from milk solids by treatment with dry ether[6] it is evident that the phosphatides are not associated with the glycerides of milk as frequently has been assumed. Further evidence of this is furnished by the fact that melted butter-fat can be freed from all but extremely minute traces of phosphorus and nitrogen by centrifugation at high speed.[7]

Most of the observers to whom reference has been made have noted the association of the ether-soluble phosphorus with the proteins of the milk and some of them have assumed that this phosphorus is present in the form of lecithalbumin. In the fol-

[4] V. Njegovan: *Biochem. Ztschr.*, liv, p. 78, 1913.

[5] N. A. Brodrick-Pittard: *ibid.*, lxvii, p. 382, 1914.

[6] Compare Burow: *loc. cit.*

[7] T. B. Osborne and A. J. Wakeman: this *Journal*, xxi, p. 91, 1915.

lowing pages we will show that, as Brodrick-Pittard assumed, milk does, in fact, yield two phosphatides, one of which contains nitrogen in the ratio of 1 : 1, yields choline containing at least one-half of the nitrogen, fatty acids and glycerophosphoric acid in proportions corresponding approximately to a stearyl-oleyl-lecithin of the structure commonly assumed for this substance. This phosphatide consequently to a certain degree resembles the typical lecithin of the textbooks, the structure of which, however, has been assumed on evidence which makes the generally accepted formula seem probable, though by no means certain.

The other milk phosphatide contains nitrogen and phosphorus in the ratio of 2:1, belongs to the group of diaminophosphatides, and is apparently similar to, possibly identical with, the diaminophosphatide obtained from egg-yolk,[8] ox[9] and horse[10] kidneys, and other animal organs. Since none of these substances has been sufficiently studied in respect to its structure, their relationship cannot yet be determined. These diaminophosphatides become insoluble in ether after precipitating with acetone the ether solution of the residue obtained by evaporating the alcoholic extract; can be crystallized from alcohol; are only slightly hygroscopic; do not change color on exposure to light and air; form snow-white powders with no wax-like properties; are soluble in chloroform; and form alcoholic solutions ne tral to litmus, which are precipitated by lead acetate or cadmium chloride. Stern and Thierfelder's compound melted at 169–170°; our most carefully recrystallized fractions as high as 190° (uncorrected), or at nearly the same temperature as that of the carnaubon described by Dunham and Jacobson; namely, 189°.

Both the monamino- and the diaminophosphatide which we have isolated from milk were obtained from alcoholic extracts of the coagulum which results from boiling the milk serum, after the casein had been precipitated with dilute hydrochloric acid.

[8] M. Stern and H. Thierfelder: *Ztschr. f. physiol. Chem.*, liii, p. 370, 1907.

[9] E. K. Dunham and C. A. Jacobson: *ibid.*, lxiv, p. 303, 1910.

[10] H. MacLean: *Biochem. Jour.*, vi, p. 333, 1911–12. See this paper for a discussion of the diaminophosphatides and the possibility that the phosphatide from the ox kidney may be a diaminophosphatide and not a triaminophosphatide.

It is probable that we have thus obtained only a part of the phosphatides of milk; for if the milk serum is neutralized by milk of lime and the resulting precipitate is filtered off, the coagulated lactalbumin obtained by boil ng the acidified filtrate is free from phosphorus. Furthermore, we have evidence that only a part of the protein precipitated by lime is coagulated when the serum is boiled without such previous neutralization. This point requires further investigation.

EXPERIMENTAL.

In preparing lactalbumin from about 3000 liters of cow's milk the casein was precipitated by adding 1 per cent hydrochloric acid until a sharp separation occurred. After straining out the casein on cheese-cloth the somewhat turbid solution was filtered through a dense felt of paper pulp. The clear filtrate, which showed only a faint opalescence, was then heated rapidly, and, after boiling for five minutes, the coagulum was filtered out and washed with hot water until the washings gave no reaction for chlorides or lactose. The coagulum was then freed from most of the adhering water by pressing into a solid cake with a hydraulic press.

This cake was ground with strong alcohol in a Nixtamal mill to a fine suspension and digested with alcohol for some days. After filtering and pressing, this process was repeated. The press cake was then again ground with strong alcohol and heated to boiling in a steam jacketed kettle, filtered out, pressed, and this treatment repeated until the hot alcohol ceased to leave more than a slight quantity of residue when a portion was evaporated. The coagulum thus extracted, when air-dried, weighed 12 kilograms. The volume of the alcoholic extract was about 325 liters and, on evaporating a portion, left a residue equal to 184 grams in the whole solution, or 1.5 per cent of the coagulum.

The alcoholic extract was concentrated under diminished pressure to about one-tenth of its volume, and the concentration further continued at a low temperature with additions of absolute alcohol until most of the water was removed. The residual syrupy liquid after standing some weeks yielded a considerable crystalline deposit consisting mostly of lactose and sodium chloride, which

doubtless had been adsorbed by the lactalbumin and was set free on heating with alcohol. This was filtered out and the alcohol largely removed by concentrating the filtrate at a low temperature *in vacuo*. The residue was taken up in ether and shaken out with water. After thus washing with water the ether was removed by distillation, leaving a residue weighing 116 grams.

This was extracted with acetone in which 37 grams failed to dissolve after repeated treatments. This residue, which had all the physical characteristics of "lecithin," was equal to 0.31 per cent of the coagulum from which it originated. The substance insoluble in acetone was treated with pure ether, but was found to be only partly soluble therein, whereas before removing the acetone-soluble substance it was completely soluble. The insoluble substance was snow-white and so finely divided that it could not be filtered out. It was accordingly separated and washed by subsidence from ether. After drying over sulphuric acid in a vacuum it weighed 8 grams.

From its solution in a little warm absolute alcohol a semi-crystalline separation in the form of microscopic balls occurred on cooling. By repeated crystallization about one-half of this substance was obtained as a product which above 169° (uncorrected) turned brown and softened; began to melt at 184° and completely melted with decomposition at 190° to a clear brown liquid. This substance contained 2.59 per cent of phosphorus and 2.45 per cent of nitrogen, giving a ratio of P : N equal to 1 : 2.09. The unhydrolyzed substance absorbed 29.8 per cent of iodine. From this it would appear that the compound is a diaminophosphatide, but we have postponed further attempts to determine its nature until we secure a larger amount.

The crude phosphatide soluble in ether, which had been obtained in two lots, was analyzed for phosphorus and nitrogen with the following results:

		A	B	AVERAGE *per cent*
I.	P	2.401	2.455	2.428
	N	1.12	1.05	1.085
Ratio N : P = 1 : 1.01				
II.	P	3.176		
	N	1.37	1.41	1.39
Ratio N : P = 1 : 1.03				

These preparations were united and purified by twice dissolving in ether, and precipitating with an excess of acetone, which removed 7.7 grams. The last precipitate was cooled to $-19°$, ground to a powder under cold acetone, filtered out by suction and freed from acetone in a vacuum over sulphuric acid at a low temperature. The dry preparation which weighed 10.8 grams, formed a slightly brownish colored powder which was very hygroscopic. On account of the rapidity with which it absorbed moisture no attempt was made to recover the substance quantitatively.

This purified phosphatide was analyzed for nitrogen and phosphorus with the following results:

	A	B	AVERAGE *per cent*
N	1.817	1.802	1.81
P	3.92	3.82	3.87

Ratio N : P = 1 : 0.97

These analyses agree very nearly with those calculated for a stearyl-oleyl-lecithin; namely, P 3.84 and N 1.74 per cent. Since ultimate analyses of substances of high molecular weight give little evidence respecting their constitution, we have instead attempted to determine the products of hydrolysis of this preparation and their relative proportions. Accordingly 5.1564 grams were boiled with 200 cc. of water containing 25 grams of crystallized barium hydroxide. The phosphatide was dissolved in a mixture of alcohol and ether and this solution added gradually to the boiling baryta solution. After cooling, the insoluble barium soap was ground up with the baryta solution and the residue again boiled to complete the hydrolysis of any phosphatide which might have escaped the action of the baryta by becoming enclosed in the pasty mass of soaps. After cooling to 0° the insoluble soap was filtered out and washed with cold water. The solution was acidified with hydrochloric acid and the barium removed by an equivalent quantity of sulphuric acid.

The filtrate from the barium sulphate was concentrated *in vacuo* and the water was removed by repeatedly adding absolute alcohol. Choline was precipitated by platinic chloride, 0.8102 gram of choline platinum chloride being obtained, equivalent to 0.3176 gram of choline, or about 40 per cent of the quantity calculated for stearyl-oleyl-lecithin, or 39.5 per cent of the nitro-

gen of the preparation hydrolyzed. During the hydrolysis alkaline vapors and an odor of trimethylamine were evolved, showing a decomposition which to some extent accounts for the low yield of choline. In view of the known difficulties encountered in recovering choline quantitatively this result cannot be regarded as showing the actual amount of choline present in this phosphatide.[11]

By recrystallizing from water the precipitate of choline platinum chloride was obtained in characteristic crystals which on ignition gave the odor of trimethylamine and 31.47 per cent Pt; calculated for $(C_5H_{14}ONCl)_2PtCl_4$ = 31.67 per cent. The filtrate from the choline platinum chloride precipitate was freed from platinum with hydrogen sulphide, concentrated *in vacuo* to small volume, water removed by frequent additions of absolute alcohol and distillation. The residue was dissolved in water and found to give no precipitate of ammonium phosphomolybdate nor of barium phosphate. Barium hydroxide was added to alkaline reaction, the excess of barium removed by carbonic acid, the solution filtered hot, and concentrated *in vacuo* to dryness. The dry residue of barium glycerophosphate weighed 1.74 grams = 18.84 per cent of glycerophosphoric acid; calculated for stearyl-oleyl-lecithin 21.35 per cent. The identity of the glycerophosphate was established by recrystallizing three times from alcohol and igniting the product dried at 106°.

I. 0.1258 gm. substance gave 0.0893 gm. barium pyrophosphate = 43.46 per cent of barium, or 9.80 per cent of phosphorus in the glycerophosphoric acid.

II. 0.1700 gm. substance gave 0.1208 gm. barium pyrophosphate = 43.50 per cent of barium, or 9.83 per cent of phosphorus in the glycerophosphoric acid.

Calculated for $C_3H_7PO_6Ba + \frac{1}{2} H_2O$ = Ba 43.42, P 9.81 per cent.

The identity of the barium pyrophosphate was established by converting into the sulphate, whereby 0.1248 gram was obtained, equivalent to 0.0734 gram Ba = 0.1200 gram barium pyrophosphate; weighed 0.1208 gram.

The barium soaps, which separated on hydrolyzing the phosphatide, were decomposed with dilute sulphuric acid, and the

[11] Compare H. MacLean: *Ztschr. f. physiol. Chem.*, lix, p. 223, 1909.

fatty acids dissolved in ether. After evaporating off the ether the residue weighed 3.4177 grams, equal to 66.3 per cent. Calculated for stearyl-oleyl-lecithin 70.2 per cent. The fatty acids thus obtained were separated by crystallization from alcohol into two fractions, one of which was solid, the other fluid at room temperature. These were converted into the lead salts which were extracted with ether. The lead salt insoluble in ether was decomposed with alcoholic hydrochloric acid, and the fatty acids were shaken out of the aqueous solution with ether. The ethereal solution left on evaporation a colored residue. This was recrystallized from alcohol until only a small quantity remained which melted at 65° and solidified at 63°. The solid fatty acid was evidently a mixture which, owing to the small amount of substance available, could not be separated into its constituents.

The lead salt, soluble in ether, was decomposed in the same way and yielded a dark colored liquid which solidified at about 0°, and, when tested by the Hanus method, absorbed 85.7 per cent of iodine. Oleic acid absorbs 89.84 per cent of iodine. The iodine absorption of the unaltered phosphatide was 42.2 per cent, which is much higher than that calculated for a stearyl-oleyl-lecithin; namely, 31.6 per cent. We thus identified choline, glycerophosphoric acid, and two fatty acids in quantities approximating those calculated for a typical "lecithin." There can therefore be no doubt that milk contains a small amount of a lecithin-like substance which separates with the coagulum when the acidified milk serum is boiled. We have reason to believe that the filtrate from this coagulum also contains an additional small amount of phosphatide, but further investigation is required before we can make this certain.

Since no special precautions to avoid exposure to air and light had been taken during the preparation of the phosphatides just described, owing to the fact that their presence in the alcoholic extract of the coagulated milk protein had not been foreseen, another lot of alcohol washings of 3139 grams of coagulum was worked up, excluding, as far as possible, the action of both air and light. The accumulated washings were kept in closed tinned milk cans and concentrated in a metal vacuum still, water being removed by additions of strong alcohol as the extract became more concentrated. The residue was taken up in ether. The ethereal

solution was washed with water in a separatory funnel covered with a black cloth, concentrated to small volume, and the clear solution poured into acetone. The precipitated phosphatides were treated with ether, and the insoluble white diaminophosphatide was separated in the same way as in making the first preparations. Of the diaminophosphatide 10.1 grams, and of the monaminophosphatide 13.0 grams, were obtained. The latter contained 1.87 per cent of nitrogen and 3.89 per cent of phosphorus. P : N = 1 : 1.067. The iodine absorption was 52.2 per cent which is much higher than that found in the first preparation, namely, 42.2 per cent, doubtless due to protection from oxidation during the preparation of the substance.

Since the first preparation was unsuitable for identification of the fatty acids, 9.5868 grams of the phosphatide dried to constant weight *in vacuo* over sulphuric acid were boiled with 500 cc. of water containing 10 cc. of concentrated hydrochloric acid for about six hours. The solution was shaken out with ether, the ether solution concentrated and poured into acetone in order to detect the presence of unhydrolyzed phosphatide. The small amount of precipitate was dissolved in a little ether and added to a dilute solution of hydrochloric acid and boiled for two and a half hours. The fatty acids were shaken out with ether as before. The ethereal solutions containing the fatty acids were evaporated and left a residue weighing 7.083 grams, equal to 74 per cent of the phosphatide, or considerably more than the 66 per cent obtained by hydrolyzing the first preparation with baryta. The difference is doubtless wholly experimental, as difficulty was encountered in decomposing the barium soaps which would easily lead to loss. The iodine absorption of these mixed fatty acids was 74.4 per cent, about five-sixths that of oleic acid.

Of the fatty acids 5.2 grams were converted into the lead soaps according to Farnsteiner's method.[12] The lead soap insoluble in benzol weighed 1.3364 grams equal to 0.776 gram of stearic acid. The fatty acid obtained from this lead salt weighed 0.7503 gram, showing the molecular weight of the acid to be approximately that of stearic acid, since some loss doubtless occurred in isolating it. This is equal to only 15 per cent, or about one-sixth, of the

[12] K. Farnsteiner: *Ztschr. f. Untersuch. d. Nahrungs- u. Genussmittel*, i, p. 390, 1898.

total fatty acids. This solid fatty acid absorbed no iodine. When recrystallized from alcohol, fractions were obtained which melted from 62° to 65° indicating a mixture of saturated fatty acids. The amount of material available was not sufficient to enable us to isolate any definite acid, the nature of which must, therefore, remain for future investigation.

The benzol soluble lead soap when decomposed yielded 4.195 grams of fatty acids, equal to 80.7 per cent, or five-sixths of the total fatty acids. The iodine absorption of the unsaturated fatty acids was 87.2 per cent, or about the amount usually obtained for oleic acid under conditions where oxidation is not wholly excluded.

The acid solution of the products of hydrolysis, from which the fatty acids had been removed, was concentrated and the water removed by additions of alcohol and evaporation. The residue was taken up in absolute alcohol, with the addition of a little alcoholic hydrochloric acid, and the choline precipitated with an alcoholic solution of platinic chloride. The precipitate of choline platinum chloride weighed 1.3496 grams. The filtrate was evaporated to dryness, the residue taken up in absolute alcohol, containing a little hydrochloric acid and more platinum chloride added. After standing over night, a precipitate separated which weighed 0.1730 gram. Four repetitions of this process yielded 0.1193, 0.3036, 0.3310, and 0.1885 gram, making the total amount of crude platinum salt 2.4627 grams equal to 0.9654 gram of choline.

The united precipitates were crystallized from water, and the choline platinum chloride was obtained from each in characteristic form. The crystals were extracted with absolute alcohol which removed a little amorphous matter. The purified choline platinum chloride weighed 2.0430 grams, equal to 0.8010 gram of choline, or 8.35 per cent of the phosphatide. When once recrystallized it contained 31.70 per cent of platinum; calculated for $(C_5H_{14}ONCl)_2PtCl_4$ = 31.67 per cent.

The choline thus accounted for contained nitrogen equal to 51.5 per cent of the nitrogen of the phosphatide. The great difficulty encountered in causing this platinum salt to separate was surprising, and it is very probable that all of the choline was not isolated from the solution.

To determine whether or not this preparation of the phosphatide contained amino-nitrogen 1.1703 grams were boiled with 0.8 per cent hydrochloric acid for several hours, the fatty acids removed by filtration, and the concentrated solution treated according to the Van Slyke method. The nitrogen evolved measured 14.45 cc. at 21° and 745 mm., equal to 0.00802 gram, or 36 per cent of the nitrogen of the phosphatide.

It must remain for future investigation to show whether this preparation of the ether-soluble milk phosphatide is a mixture of two substances, one yielding choline and the other a base which contains amino-nitrogen, or is a complex phosphatide containing both bases. The fact that the saturated fatty acid fraction had no definite melting point indicates that the preparation is a mixture. An attempt will be made to fractionate this substance in order to determine whether or not the two bases belong to different substances, and also to secure further evidence as to the nature of the fatty acids which it contains.

An attempt to determine the molecular weight, which Dr. Carl O. Johns kindly made for us, led to the unexpected result that when dissolved in benzol the freezing point of the solution was raised about 0.07 of a degree. This fact could be explained by crystallization of the phosphatide. This was shown to be the case by allowing the crystals to settle, and drawing off the clear solution with a pipette. When 2 cc. of the solution thus drawn off were evaporated the residue weighed 0.038 gram, whereas the same amount of the solution containing the melted crystals weighed 0.068 gram. We hope to take advantage of this property of the phosphatide in order to obtain it in crystalline form.

To determine whether these phosphatides could be extracted from the coagulated milk protein by direct extraction with ether, or became soluble therein only after previous treatment with alcohol, we dried a quantity of the well washed coagulum at a low temperature and ground it to as fine a powder as we were able. Of this material 242 grams were kept for several days, with frequent shaking, under a liberal quantity of pure ether. The ether extract contained 0.66 gram of oil which was wholly free from phosphorus and completely soluble in acetone. A second extraction removed only 0.127 gram, which was likewise soluble in acetone, making 0.33 per cent of fat extracted by ether.

The coagulum was then digested repeatedly with absolute alcohol until the amount of substance extracted after long treatment became relatively small. The residues left by evaporating the alcohol extracts weighed 4.64 grams in all, equal to 1.9 per cent of the coagulum. The solution of this residue in a little ether when poured into acetone gave a precipitate which weighed 2.23 grams and left 2.41 grams of substance in solution, equal to 1 per cent of the coagulated milk protein.

The precipitate when treated with ether showed the presence of the white diaminophosphatide which is insoluble in ether. This was separated as completely as possible from the monaminophosphatide by repeated solution of the latter in ether and precipitation with acetone. By this procedure we obtained 1.11 grams of the monaminophosphatide containing 3.87 per cent of phosphorus and 1.12 grams of the diaminophosphatide, which contained 2.19 per cent of nitrogen and when recrystallized from alcohol melted with decomposition at 188–189° (uncorrected).

What relation these substances have to the protein is difficult to determine. That some form of combination exists must be assumed, for they become soluble in ether only after the protein is treated with alcohol, and, furthermore, before coagulating the protein by heat, they are freely soluble in water. It is probable that these phosphatides are not combined with the lactalbumin which is the chief constituent of the coagulum, but with another protein, for we have found that when the filtrate from the casein is neutralized with milk of lime the acidified filtrate from the precipitate yields a coagulum which is free from phosphorus. Since the precipitate produced by thus neutralizing contains protein it is probable that the phosphatides are also contained therein as a lecithalbumin containing a relatively large proportion of phosphatide similar to ovovitellin of the egg-yolk.[13]

[13] Compare T. B. Osborne and G. F. Campbell: *Jour. Am. Chem. Soc.*, xxii, p. 413, 1900.

A RAPID METHOD FOR DETERMINING CALCIUM IN URINE AND FECES.

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The tedious character of the methods hitherto available for the determination of calcium in the presence of magnesium and phosphates is undoubtedly in part responsible for the scantiness of the literature on the metabolism of this element. The immediate object of this investigation was to procure a more rapid method for the determination of calcium in the urine and feces of children, so that more exact information might be obtained with reference to such specific metabolism disorders as are believed to exist in rachitis and other more or less similar conditions. It was thought that by making use of the turbidity principle already employed for the determination of proteins, acetone bodies, etc., the necessary speed might be attained.

At first an attempt was made to read the cloud caused by the precipitation of calcium oxalate in the urine itself, the urine being first rendered colorless by shaking with repurified blood charcoal and filtering. As was found by analysis, the charcoal did not adsorb calcium from the urine. The results were, however, uniformly too low, and on examination of the precipitate under the microscope the reason for this became apparent. In precipitation from pure aqueous solutions of calcium salts the crystals of the oxalate come down in feathery clusters which would naturally form a very opaque suspension; when, on the other hand, magnesium is present the crystals are in compact, chunky masses and the resulting suspension is comparatively clear. It seemed necessary, therefore, to separate the calcium from the magnesium as oxalate, according to McCrudden's method. This may most easily be done by means of the centrifuge. After centrifuging,

the calcium oxalate was dissolved in dilute hydrochloric acid, an excess of ammonium oxalate added, and this solution tried against a standard of the same character, both being made alkaline simultaneously. When the standard and the urine were nearly alike in calcium content this procedure gave good results, but when there was any considerable variation not only were the clouds not quantitative, but the colors were markedly different, so that they could not be matched. The addition of ammonium chloride and the use of a number of substances, particularly protective colloids, such as egg-white, gum arabic, slippery elm, tragacanth, acacia, starch, and dextrin, were tried with some improvement, but without complete success.

Precipitation of the calcium as soap was therefore determined upon, the reagent chosen being an aqueous solution of potassic ricinate. The ricinate is superior to oleates, stearates, and mixtures in many ways. It is easily prepared, gives a clear, nearly colorless solution, and does not become cloudy on standing (at least for a number of days). Luckily the calcium soap clouds may be read quantitively to about the limits of the colorimeter. The colors match well and, the solution being somewhat viscous, the precipitate does not settle out for several hours.

The completed method, a description of which follows, takes only twenty-five minutes after the specimen has been prepared for analysis. A small quantity of urine may be used, which is occasionally convenient in the case of infants. There are three main steps: (1) isolation of the calcium as calcium oxalate; (2) solution of the calcium oxalate in dilute acid; (3) precipitation of the calcium as soap, and comparison of the cloud so formed with the cloud of a standard solution by the Duboscq colorimeter.

A description of the treatment of urine will be given and then the modifications necessary for feces.

The first step is done exactly as in McCrudden's[1] excellent method, from which this part of the procedure is copied, but on a small scale.

If the urine is alkaline, make slightly acid.

Filter (otherwise the calcium oxalate precipitate is hard to centrifuge out).

[1] F. H. McCrudden: this *Journal*, x, p. 187, 1911–12.

Make just alkaline with concentrated ammonium hydrate, added drop by drop. Make just acid with concentrated hydrochloric acid (specific gravity 1.20). If the urine is warmed by the neutralization, it should now be cooled. For every 100 cc. of urine add five more drops of acid. If the cloud of phosphates produced by making alkaline is easily seen, it may be used as an indicator,—otherwise use litmus paper. This cloud is also a help in deciding how much urine to take. If it is heavy, 5 cc. will probably be enough; if it is light take 8 cc., 10 cc., or more. Usually with normal adults on a mixed diet 8 cc. will give the best results.

Pipette 5, 8, or 10 cc. into a small Erlenmeyer flask. Add 1 cc. of 2 per cent oxalic acid solution. Add 1 cc. of 10 per cent sodium acetate solution (10 grams of crystallized sodium acetate to 100 cc. of water). 2 cc. are used in the case of feces.

Stopper and shake well for ten minutes by the watch. This shaking, as was shown by McCrudden, takes the place of standing over night. The precipitation is not done in a centrifuge tube, as there is a tendency for the precipitate to cling loosely to the walls. Rinse the stopper with a little 0.5 per cent ammonium oxalate solution. Pour into a centrifuge tube. Rinse the flask with 2 cc. of 0.5 per cent ammonium oxalate. Centrifuge until the supernatant liquor is perfectly clear (two to three minutes).

Pour off, being careful not to disturb the precipitate.

Wash the precipitate with 10 cc. of 0.5 per cent ammonium oxalate solution. Centrifuge and decant. With the help of a stirring rod dissolve the precipitate in 5 cc. of 5 per cent hydrochloric acid. If it dissolves with difficulty the tube may be placed in hot water for a minute or two and afterward cooled. Pour into the original Erlenmeyer flask. Rinse the tube and stirring rod with 5 cc. of distilled water. Agitate the liquid in the flask for a moment to dissolve any precipitate clinging to the walls. In another flask take 10 cc. of a standard solution of calcium oxalate in 2.5 per cent hydrochloric acid, containing 1.5 mg. of calcium per 10 cc. [0.5475 gram of calcium oxalate, (CaC_2O_4, 1 H_2O) in a liter of 2.5 per cent hydrochloric acid]. From a pipette from which the tip has been broken, run into each flask 20 cc. of the potassic ricinate solution to be described,

agitating the flask meanwhile. Mix. Allow to stand for two minutes. Read the clouds by the Duboscq colorimeter in the usual way.

If it is found that the amount of urine taken contains less than 0.75 mg. or more than 2.5 mg. of calcium, the readings are no longer quantitative and another determination must be done with a volume of urine which will give something between these figures. This difficulty cannot be overcome by diluting either the standard or the unknown after precipitation, as the results will not be accurate. If 10 cc. of urine are taken and the unknown is set at 15 mm., the reading of the standard will equal the number of mg. of calcium in 100 cc. of urine. The deeper clouds may be read at 10 mm., and the lighter ones at 20 mm. In any case where the unknown is set at a given height and the standard read against it, the formula will be:

$$\frac{\text{Reading of the standard} \times 1.5}{\text{Height at which the unknown was set} \times \text{No. of cc. urine taken}} = \text{mg. of calcium in 1 cc.}$$

Should the urine be very much concentrated so that 5 cc. contain more than 2.5 mg. of calcium, it should be diluted. If, on the other hand, it is so dilute that an amount containing 0.75 mg. will not go into the centrifuge tube, the necessary quantity is precipitated in a flask (a proportionately greater volume of acetate should be used) and centrifuged in successive portions. That is, a tubeful is centrifuged, the supernatant liquor poured off, the tube again filled from the flask, and so on until the precipitate from the whole amount is collected. Large centrifuge tubes with round bottoms, such as are used in some other processes, are not suitable, since part of the precipitate will always come away when pouring off.

In this calcium ricinate precipitation the clouds continue to grow darker for fifteen to twenty minutes, but in two solutions of different strength (containing between 0.75 mg. and 2.5mg. of calcium) this increase of turbidity is parallel. Therefore if the solutions are mixed as nearly as possible together, they can be read correctly in one or two minutes, making it unnecessary to wait the full fifteen minutes. A few points in the management of the colorimeter may here be mentioned, which add to the

accuracy of turbidity work, and which are sometimes slighted. Since light falling laterally on the cups of the colorimeter is in some measure reflected to the eye by particles in suspension, the shade, which for convenience is usually discarded when colors are being matched, must always be used. An artificial light containing much yellow or red is not permissible. Daylight from a clear sky is the best. The colorimeter must be kept perfectly clean and should always be tested with the standard in both cups before attempting to read the unknown. If the operator has been accustomed to reading colors some practice is necessary before he can work accurately with clouds. It should be remembered that, with the same suspension, a deeper column appears blue, while the shorter column looks brown. When brought to the same level, however, the colors should be identical. Distilled water must be used throughout the method.

Potassic ricinate is prepared as follows: Dissolve 15 grams of potassic hydrate in a mixture of 25 cc. of water and 100 cc. of alcohol. Warm and add 100 cc. of castor oil. Shake well. Heat under a reflux condenser on the boiling water bath until a sample dissolves in water with no free oil showing. This usually takes about seven hours. Keep this as a stock solution. Of the stock solution pipette 35 cc. into a flask. Add 965 cc. of distilled water in which have been dissolved 9 grams of sodic hydrate. The reagent should be nearly colorless and perfectly clear. It must be made up fresh from the stock solution once a week. No attempt was made to remove either the glycerin or the slight excess of alcohol from the ricinate, as experiment showed they do not interfere. The excess of sodic hydrate is added to neutralize the acid in which the calcium oxalate is dissolved and which would otherwise throw down fatty acids, thus obscuring the soap cloud. Potassic hydrate does not give good results. When working with feces a little more preparation of the substance is necessary. After thorough mixing, a small portion, 5 or 6 grams, is taken, weighed, moistened with concentrated sulphuric acid, and burned to a white powder in a small silica dish. Heat gently at first, as there is considerable frothing. The residue is washed into a 100 cc. volumetric flask with 50 cc. of 10 per cent hydrochloric acid. The solution is made up to volume, well shaken, and filtered. Of the filtrate 50 cc. are taken and made up to 100

cc. with distilled water. This solution is then analyzed exactly as urine, except that 2 cc. of sodium acetate are used instead of 1 cc. Without the excess of acetate the precipitate is very fine and difficult to handle. If for any reason the output of calcium is far from the normal, the volume up to which the ash solution is made can be varied at will.

The following table gives the results of ten analyses of urine by the new method, checked by the method of McCrudden.

NEW METHOD Calcium per 100 cc.	METHOD OF MCCRUDDEN Calcium per 100 cc.
mg.	*mg.*
16.5	16.6
26.4	26.4
19.5	19.9
35.0	34.9
11.6	12.2
27.3	27.2
19.0	19.1
34.2	34.1
15.1	14.9
30.1	29.9

THE FUNCTION OF THE LIVER IN UREA FORMATION FROM AMINO-ACIDS.

By B. C. P. JANSEN.

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(Received for publication, March 25, 1915.)

The interesting experiments of Folin,[1] Van Slyke,[2] and Costantino,[3] have shown that the amino-acids resulting from protein digestion in the intestines are probably absorbed, without undergoing any change, by the wall of the intestines, and are distributed with the blood streams in the various tissues of the body.

The question therefore arises: What becomes of the amino-acids taken up by the tissues? For in a very short time the same quantity of nitrogen that is taken in the food is removed from the body, chiefly in the form of urea.

One of the first questions that arises is: Where does the transformation of amino-acids into urea take place? The answer is important not only theoretically and clinically, but also technically; for if we know in which organ or organs amino-acids are transformed into urea, we may study the transformation more directly. Since the classical experiments of von Schroeder in 1882, it is now generally accepted that ammonium salts are transformed into urea in the liver. It was not until 1896, however, that Salaskin undertook a series of experiments on the fate of amino-acids perfused through a surviving liver. He found that they also were transformed into urea. These results have recently been attacked by Folin, and especially by two of his pupils, Fiske and Karsner.

[1] O. Folin and W. Denis: especially First to Third Papers, this *Journal*, xi, pp. 87 and 161; xii, p. 141, 1912.

[2] D. D. Van Slyke and G. M. Meyer: *ibid.*, xii, p. 399, 1912; xvi, pp. 197 and 213, 1913.

[3] A. Costantino: *Biochem. Ztschr.*, li, p. 91, 1913; lv, p. 402, 1913.

Fiske and Karsner have repeated the experiments of von Schroeder and of Salaskin. They have perfused surviving livers of cats and of rabbits with ammonium carbonate or with glycocoll. In the first experiments, with ammonium carbonate, they obtained an increase of the urea content of the perfusing fluid; on the contrary, when glycocoll was added to the perfusing fluid an increase of the urea could not be detected. In these researches they have used the quantitative methods worked out in the laboratory of Folin, which naturally will be exact. But the method of perfusion, which was practised by them, seems to me not to be very efficient, especially regarding the arterialization of the blood. It may be noted that in a later publication the authors established that much more ammonium carbonate can be transformed into urea when pure oxygen is used for arterializing the blood.[4] In this publication, experiments with amino-acids passed through the liver are not mentioned.

I have repeated the experiments of Fiske and Karsner, but have used the perfusion apparatus of Mandel-Embden, whereby care is taken to assure a good arterialization of the blood.

Technique.

The dogs were bled to death from the femoral arteries or from the carotid arteries under ether narcosis. The liver was taken out of the body as soon as possible (ten to twenty minutes) and brought into the apparatus. The first 300 to 400 cc. of the perfusing fluid were cast away to wash out the fibrinogen from the liver. I then waited till all the fluid had flowed once through the liver. Then a portion of 50 or, usually, 100 cc. was measured exactly by a pipette, and immediately deproteinized according to Schenck.

The liver was then perfused for half an hour without any addition. Then again a portion of 50 or 100 cc. was taken with the purpose of determining the amount of urea formed out of "ureogenic" substances possibly present in the perfusing fluid. At the same time a solution of an amino-acid was added. After another half hour and after an hour 50 or 100 cc. were again taken.

[4] C. H. Fiske and H. T. Karsner: this *Journal*, xviii, p. 381, 1914.

To determine the urea content of the different portions, I have chosen the urease method, which seems to me to be the most simple and to give the most exact results.

A method has been worked out by Marshall[5] to determine the urea in the blood directly. But I feared that in the strong albuminous ferment- and bacteria-containing fluid, slight alterations in the ammonia and urea content might appear, especially since the blood generally could not be worked upon on the day of the perfusion. Therefore, as mentioned above, I immediately deproteinized the blood by adding an equal volume of 2 per cent hydrochloric acid and an equal volume of 6 per cent mercuric chloride solution. (In the original method of Schenck for 100 cc. of blood, 200 cc. of hydrochloric acid and 200 cc. of sublimate solution are used; the fluid is then diluted six times; I found that 100 cc. of hydrochloric acid and 100 to 125 cc. of sublimate solution were always sufficient to obtain a clear filtrate.)

The following day the solution is filtered and from the filtrate an exactly measured portion (usually 200 cc.) is made alkaline with sodium carbonate and the ammonia distilled off *in vacuo* into a measured quantity (usually 1 cc.) of 0.1 N sulphuric acid. To prevent foaming 3 to 5 cc. of amylalcohol are added. When the ammonia is removed the remaining fluid is neutralized with acetic acid to rosolic acid (orange-colored)[6] and then urease is added. Sometimes an aqueous extract was used;[7] sometimes finely powdered soya beans were suspended in the fluid.[8] The mixture was now digested so long at 40° that I was sure all urea was hydrolyzed into ammonium carbonate. Then a large quantity of sodium carbonate and again some amylalcohol was added, and the ammonia distilled *in vacuo* into a measured quantity of 0.1 N sulphuric acid. The excess of sulphuric acid was titrated with 0.1 N caustic soda solution, whereby rosolic acid was used as indicator.

The results obtained are given in the following table. A more detailed account of these experiments and of the literature will

[5] E. K. Marshall, Jr.: *ibid.*, xv, p. 487, 1913.

[6] Marshall: *ibid.*, xvii, p. 357, 1914. D. D. Van Slyke and G. Zacharias: *ibid.*, xix, p. 181, 1914.

[7] Marshall: *ibid.*, xiv, p. 283, 1913.

[8] Plimmer and Skelton: *Biochem. Jour.*, viii, p. 70, 1914.

soon be published in the *Archives néerlandaises des sciences exactes et naturelles.* The following points in the table may be noted. In the first place the values obtained for the ammonia content of the perfusing fluid are not mentioned. These values are, however, very low and do not alter very much in the course of the experiment; in many cases they increase a little; in many other cases the increase was not measurable; in the first portion of blood the content was usually about 0.5 mg. per 100 cc. of fluid; in the last, at the most 1 to 2 mg. Exceptions are Experiments IX and XV; in the first the ammonia content rose to 5.7 mg; in the second to 4.3 mg. per 100 cc. of perfusing fluid.

In the experiments with the cats, the values obtained for the increase of the urea content when the liver was perfused with an amino-acid were a great deal higher than those obtained by Fiske and Karsner when they used ammonium carbonate.

In the experiments where leucine was added to the perfusing fluid, the yield of urea is very small, and not much higher than it would have been had nothing been added. It is remarkable that Salaskin also in some experiments with leucine did not obtain good results. The explanation given by Salaskin, *viz.*, that the leucine crystallized out in the capillaries, does not seem to me to be very probable, when we take into consideration the large quantity of fluid in which it is dissolved.

Embden has also encountered difficulties when he used leucine to investigate the formation of acetyl-acetic acid from amino-acids in the liver. According to Embden the non-natural *d*-leucine gives a much better yield than the *l*-leucine obtained from casein.

The blank Experiments XIII and XIV, and properly all the first half hours of the other experiments are remarkable, because they do not agree with the findings of Salaskin and of von Schroeder; these investigators could not detect a formation of urea, when nothing was added to the perfusing fluid. It seems possible to explain this difference from the fact that the blood, which was used in Experiments XIII and XIV, was much diluted with Ringer's solution, to which no urea was added. It is therefore possible that this fluid washed urea out of the liver. But this cannot be the only explanation. According to Marshall, the liver of a normally fed dog contains about 30 mg. of urea per 100 gm. of liver; in Experiment XIV, however, 180 mg. of urea

	Urea in 100 cc. of perfusion liquid				Total quantity of urea formed in last hr.	Mean rate of blood flow per min.
	After 0 min.	After 30 min.	After 60 min.	After 90 min.		
	mg.	mg.	mg.	mg.	mg.	cc.
	33.3	36.0	53.1	61.0 (after 75 min.)	520	200–300
	24.7	26.5	—	51.0 " "	441	110–140
	4.5	9.0	11.7	15.3	49.5	170–190
	6.3	9.0	12.6	16.2	42	250–300
	7.6	9.6	—	29.	137	
	9.2	10.0	20.4	44.0	176	
)0 mg.						
	14.0	14.8	22.6	29.6	125	220
	13.5	15.9	19.7	28.7	92	220
	5.8	8.0	11.2	17.2	58	160
	14.8	18.8	22.8(26.4)*	27.2(31.2)*	132	400
	—	16.8	22.0	28.0	117	360
	20.7	25.2	31.5	36.9	170	150
	13.4	—	21.2	25.2	82	400
	13.2	20.4	24.4	30.0	120	300
	15.6	17.6	23.6	34.4	157	380
	7.4	8.6	13.0	19.6	109	440
	8.4	9.6	13.2	16.8	72	320
X	27.7	32.4	35.8	38.3	86	220
	10.8	12.5	16.2	20.6	85	260
	38.9	43.9	53.3	62.3	230	200
ine	32.0	37.8	47.3	56.3	269	120
X	17.2	24.8	40.0	88 (after 150 min.)	264	400
X e	25.5	27.	28.9	31.9	29	340
X	32.9	36.7	51.5	75.6	522	240
	17.1	19.1	29.3	40.5	288	150
X	16.7	18.7	38.7	59.2	547	±150

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EXPERIMENT NO.	WEIGHT OF ANIMAL	WEIGHT OF LIVER	STATE OF NUTRITION	PERFUSION LIQUID	ADDED AFTER 30 MIN.	UREA IN 100 CC. OF PERFUSION LIQUID				TOTAL QUANTITY OF UREA FORMED IN LAST HR.	MEAN RATE OF BLOOD FLOW PER MIN.
						After 0 min.	After 30 min.	After 60 min.	After 90 min.		
	kg.	*gm.*				*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*cc.*
I (dog).........		190	Fasted at least 2 days	1700 cc. ox blood	3 gm. glycocoll	33.3	36.0	53.1	61.0 (after 75 min.)	520	200–300
II (dog).........		170	Fasted at least 2 days	1650 cc. ox blood	5 gm. ereptone	24.7	26.5	—	51.0 " "	441	110–140
III (cat).........		65	Fasted for 24 hrs.	250 cc. ox blood corpuscles + 550 cc. Ringer's solution	2 gm. glycocoll	4.5	9.0	11.7	15.3	49.5	170–190
IV (cat).........		75		250 cc. ox blood corpuscles + 650 cc. Ringer's solution	1.8 gm. leucine	6.3	9.0	12.6	16.2	42	250–300
V (cat).........		45	Fasted 2 days	300 cc. ox blood corpuscles + 600 cc. Ringer's solution	1.5 mg. ammonium carbonate	7.6	9.6	—	29.	137	
VI (cat)........	2.5	60		250 cc. ox blood corpuscles + 600 cc. Ringer's solution	0.6 gm. ammonium carbonate	9.2	10.0	20.4	44.0	176	
VII (cat)..........	2.4	60	Fasted 3–4 days	350 cc. ox blood corpuscles + 750 cc. Ringer's solution	Ereptone solution containing 200 mg. amino-acid nitrogen	14.0	14.8	22.6	29.6	125	220
VIII (cat)..........	3.0	54	Fasted 3 days	325 cc. ox blood corpuscles + 675 cc. Ringer's solution	1.6 gm. glycocoll	13.5	15.9	19.7	28.7	92	220
IX (cat)..........			Fasted 2 days	250 cc. ox blood corpuscles + 650 cc. Ringer's solution	2.5 gm. glycocoll	5.8	8.0	11.2	17.2	58	160
X (dog)........	8	210	Fasted 3 days	350 cc. ox blood corpuscles + 800 cc. Ringer's solution	4.5 gm. leucine in 150 cc. Ringer's	14.8	18.8	22.8(26.4)*	27.2(31.2)*	132	400
XI (dog)........		160	Fasted 2 days	300 cc. ox blood corpuscles +1000 cc. Ringer's solution	4 gm. leucine	—	16.8	22.0	28.0	117	360
XII (dog)........	4.25	125	Fasted 2 days	1700 cc. ox blood	1.9 gm. leucine	20.7	25.2	31.5	36.9	170	150
XIII (dog)........	9.5	250	Well fed	200 cc. dog blood + 1000 cc. Ringer's solution	Nothing	13.4	—	21.2	25.2	82	400
XIV (dog)........	9.5		Pregnant	300 cc. ox blood corpuscles + 900 cc. Ringer's solution	Nothing	13.2	20.4	24.4	30.0	120	300
XV (dog)........			Fasted 2 days	200 cc. dog blood + 1000 cc. Ringer's solution	2 gm. glycocoll	15.6	17.6	23.6	34.4	157	380
XVI (dog).........	7.5	185	Fasted 2 days	250 cc. dog blood + 1000 cc. Ringer's solution	1.1 gm. glycocoll	7.4	8.6	13.0	19.6	109	440
XVII (dog)........	6	200	Well fed	350 cc. ox blood corpuscles + 900 cc. Ringer's solution	5 gm. glycocoll	8.4	9.6	13.2	16.8	72	320
XVIII	8.5	315	Well fed	1700 cc. ox blood	2 gm. glycocoll	27.7	32.4	35.8	38.3	86	220
XIX (dog)........	6	180	Fasted 2 days	350 cc. ox blood corpuscles + 950 cc. Ringer's solution	2.2 gm. alanine	10.8	12.5	16.2	20.6	85	260
XX (dog)........	5			1600 cc. ox blood	2 gm. alanine	38.9	43.9	53.3	62.3	230	200
XXI (dog)........	11		Fasted 3 days	1700 cc. ox blood	0.75 gm. glycocoll +0.75 gm. alanine	32.0	37.8	47.3	56.3	269	120
XXII (dog)........	6.5		Fasted 7 days	300 cc. dog blood + 400 cc. blood of another dog, fasting 3 days, + 600 cc. Ringer's solution	0.7 gm. glycocoll +1 gm. alanine	17.2	24.8	40.0	88 (after 150 min.)	264	400
XXIII (dog).........	4.5	175	Well fed	150 cc. dog blood + 700 cc. Ringer's solution	0.5 gm. glycocoll +0.5 gm. alanine	25.5	27.	28.9	31.9	29	340
XXIV (dog).........	6.5	220	Fasted 7 days	1600 cc. ox blood	0.8 gm. glycocoll +1 gm. alanine	32.9	36.7	51.5	75.6	522	240
XXV (dog).........	9.5	250	Well fed	1600 cc. ox blood	2 gm. glycocoll	17.1	19.1	29.3	40.5	288	150
XXVI (dog).........	5.5	170	Fasted 7 days	1600 cc. of same blood as in the foregoing experiment	2 gm. glycocoll	16.7	18.7	38.7	59.2	547	±150

* The values in parenthesis are obtained by taking into consideration the fact that the perfusing fluid is diluted by the 150 cc. of Ringer solution in which the leucine is dissolved.

were yielded to the perfusion fluid. Only a small part of the total increase of urea could therefore be caused by washing out from the liver. Furthermore, in the experiments in which the original urea content of the perfusing fluid is high (Experiments XX, XXI, and XXIV), more urea is produced than in the experiments in which the liver was perfused with a fluid of a low urea content (*e.g.*, Experiments XVI and XVII).

Finally, it may be noted, that the quantity of urea produced in several experiments, *viz.*, 200 to 500 mg. in one hour, corresponds with 8 to 12 gm. in twenty-four hours, which is a large part of the daily urea output of a dog of 6 to 8 kg.

CONCLUSION.

Contrary to the opinion of Folin and to the perfusion experiments of Fiske and Karsner, it appears from the above experiments that the liver can play an important part in the formation of urea from amino-acids.

THE PREPARATION OF SARCOSINE.

By LOUIS BAUMANN.

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(Received for publication, May 24, 1915.)

Sarcosine is usually prepared by heating ethylmonochloroacetate with methylamine solution in a sealed tube, according to the method of Volhard.[1] Paulmann[2] prepared the acid from caffeine by decomposition with barium hydroxide; Delépine,[3] by the hydrolysis of methylaminoacetonitrile formed through the interaction of a polymer of methylenemethylimine $(CH_3 \cdot N : CH_2)_3$ with hydrocyanic acid; and Eschweiler,[4] by the reaction of methylene cyanhydrine (glycollic nitrile) with methylamine solution followed by saponification of the methylaminoacetonitrile thus formed

$$CH_2(OH)CN + NH_2CH_3 = CH_2NH(CH_3)CN + H_2O$$

$$CH_2NH(CH_3)\ CN + 2\ H_2O = CH_2NH(CH_3)COOH + NH_3$$

Recently Fischer and Bergmann[5] synthesized sarcosine hydrochloride by the methylation of para toluenesulphoglycin followed by hydrolysis of the toluenesulphonyl linkage.

While preliminary experiments were in progress which had for their aim the synthesis of sarcosine by means of the Zelinsky and Stadnikoff[6] modification of the old Strecker-Tiemann process,[7,8] an article by Heimrod[9] appeared in which the author states that he obtained sarcosine by a similar method; but no mention of the yield is made nor are analytical data recorded.

The following method, devised after considerable preliminary experimentation, is relatively simple and has proven more expeditious than any of these above enumerated.

[1] J. Volhard: *Ann. d. Chem.*, cxxiii, p. 261, 1862.

[2] W. Paulmann: *Arch. d. Pharm.*, ccxxxii, p. 601, 1894.

[3] M. Delépine: *Bull. Soc. chim.*, 3d series, xxix, p. 1198, 1903.

[4] W. Eschweiler: *Ann. d. Chem.*, cclxxix, p. 39, 1894.

[5] E. Fischer and M. Bergmann: *Ann. d. Chem.*, cccxcviii, p. 96, 1913.

[6] N. Zelinsky and G. Stadnikoff: *Ber. d. deutsch. chem. Gesellsch.*, xli, p. 2061, 1908.

[7] A. Strecker: *Ann. d. Chem.*, lxxv, p. 29, 1850.

[8] F. Tiemann: *Ber. d. deutsch. chem. Gesellsch.*, xiii, p. 381, 1880; xiv, p. 1957, 1881.

[9] G. W. Heimrod: *ibid.*, xlvii, p. 338, 1914.

1 gram molecule (67.5 grams) of methylamine hydrochloride is dissolved in 120 cc. of commercial formaldehyde solution (about 37 per cent) and to this 1 gram molecule (65 grams) of potassium cyanide dissolved in the least amount of water at 40° is added until the temperature of the mixture reaches 70°. While this temperature is maintained by means of ice the remainder of the cyanide solution is added. At the end of three hours the brownish-colored mixture, which consists of a watery and an oily layer and a sediment of salts, is extracted five times with ether in a separating-funnel, the ethereal extract dried with anhydrous sodium sulphate, then filtered, and the ether distilled off on the water bath. The last traces of ether and some of the excess of formaldehyde are removed by blowing a stream of air into the flask. The residue is then suspended in 400 cc. of water to which 125 grams of $Ba(OH)_2 . 8H_2O$ are added and the nitrile is saponified by boiling under reflux for twelve hours. During the early stage of this process care should be exercised to prevent loss by foaming (liberation of ammonia). From the cold filtered solution, to which a little ammonia has been added, the barium is removed by a stream of carbon dioxide. The barium carbonate is filtered off with suction, washed with water, and the solution evaporated, preferably *in vacuo*, to a thick syrup. This is taken up in the least amount of boiling absolute alcohol and placed in the refrigerator for twenty-four hours. The sarcosine which has crystallized is then filtered off with suction and washed with cold absolute alcohol until almost colorless. Yield: 22 grams, or 25 per cent of the theoretical. The methylamine hydrochloride required for the above synthesis is easily prepared according to the method of Brochet and Cambier[10] as follows: Ammonium chloride (500 grams) is heated with commercial formaldehyde solution (1 kilo) for three hours under reflux. The solution is then evaporated on the water bath until a good crop of crystals is obtained, cooled, and filtered. These crystals consist of unaltered ammonium chloride. The filtrate is again evaporated until crystallization occurs, cooled, and filtered. These crystals when recrystallized from boiling 95 per cent alcohol yield from 85 to 110 grams of pure methylamine hydrochloride.

[10] A. Brochet and R. Cambier: *Bull. Soc. chim.*, 3d series, xiii, p. 533, 1895.

Analytical data.

Sarcosine. 0.2912 gm. of substance (Kjeldahl) required 32.22 cc. of 0.1 normal sulphuric acid, equivalent to 0.0451 gm. of nitrogen.

Calculated for $C_3H_7O_2N$: 15.73 per cent.

Found: 15.49 per cent.

Sarcosine hydrochloride. The hydrochloride was prepared by evaporation of the substance with concentrated hydrochloric acid solution in alcohol and precipitation with ether.

0.0507 gm. of substance when dried *in vacuo* over sulphuric acid yielded 0.0575 gm. of silver chloride, equivalent to 0.0142 gm. of chlorine.

Calculated for $C_3H_8O_2NCl$: 28.28 per cent.

Found: 28.05 per cent chlorine.

Methylureidoacetic acid. The ureido acid, $NH_2.CO.N(CH_3)CH_2.COOH$, was prepared by heating 10 grams of substance with 10 grams of urea and 25 cc. of water for eight hours under reflux; then adding 100 cc. of water and 30 grams of crystalline barium hydrate, and heating on the boiling water bath for another two hours. The solution is then evaporated *in vacuo*, the residue dissolved in water and saturated with carbon dioxide, filtered, and the barium salt present in the solution decomposed with sulphuric acid. The barium sulphate is filtered off on paper-pulp, covered with kieselguhr, and thoroughly washed with hot water. When the solution is evaporated *in vacuo* crystals of the pure acid appear. Yield: 50 to 55 per cent of the theoretical; m.p. 153° (uncorrected).

0.2255 gm. of substance (Kjeldahl) required 33.8 cc. of 0.1 normal sulphuric acid, equivalent to 0.0473 gm. of nitrogen.

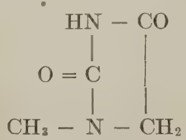
Calculated for $C_4H_8O_3N_2$: 21.21 per cent.

Found: 20.98 per cent.

0.0639 gm. of substance is neutralized by 4.7 cc. of 0.1 normal sodium hydrate solution, with phenolphthalein as an indicator.

Calculated: 4.8 cc.

Methylhydantoin. The hydantoin



was obtained by boiling the ureido acid with 5 N hydrochloric acid for three hours under reflux, then evaporating the acid and recrystallizing from hot absolute alcohol. Yield: 66 per cent of the theory; m.p. 153° (uncorrected).

0.1031 gm. of substance, dried *in vacuo* at 80° over phosphorus pentoxide, (Kjeldahl) required 17.89 cc. of 0.1 normal sulphuric acid, equivalent to 0.0250 gm. of nitrogen.

Calculated for $C_4H_6O_2N_2$: 24.56 per cent.
Found: 24.25 per cent.

0.00524 gram of the hydantoin when treated with saturated picric acid and 10 per cent sodium hydrate according to the method of Folin[11] develops a color which is equivalent to that developed by 0.00026 gram of creatinine treated in the same manner. The color intensity is therefore but 5 per cent of that of creatinine under the above conditions.

[11] O. Folin: *Ztschr. f. physiol. Chem.*, xli, p. 223, 1904.

THE QUANTITATIVE DETERMINATION OF CREATINE IN MUSCLE AND OTHER ORGANS.

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INTRODUCTION.

Existing methods for the determination of creatine in muscle and other organs are usually based on one of two procedures: the conversion of creatine into creatinine by heating either an organ extract or the entire organ with a mineral acid. The latter procedure combines hydrolysis of the tissues with conversion of the creatine.[1] It was first used by Pekelharing and van Hoogenhuyze, and has recently been employed in methods described by Baumann and by Folin.[2] The results obtained in some cases are, however, considerably higher than those secured in determinations made with the aid of extraction. This difference has been ascribed to the failure of complete removal of creatine in extracting, or to the setting free of additional amounts of this substance from higher complexes through the action of the acid. Aside from its analytical bearing, the questions here involved are of importance in their relation to the origin of creatine and creatinine. The scope of the present communication includes a study of this subject combined with an investigation of previously described procedures for the quantitative determination of creatine in muscle and organs. A description of improved analytical methods is also given.

[1] Throughout this article the term "creatine" is considered, for the sake of convenience, also to include creatinine present in the tissue as such. Results are calculated in terms of creatine.

[2] C. A. Pekelharing and C. J. van Hoogenhuyze: *Ztschr. f. physiol. Chem.*, lxiv, p. 262, 1910. L. Baumann: this *Journal*, xvii, p. 15, 1914. O. Folin: *ibid.*, xvii, p. 475, 1914.

The creatine content of muscle.

Myers and Fine[3] making use of extraction, and Folin and Buckman[4] employing the Folin hydrolysis method, have reported a series of creatine analyses of muscle of different species. The average values obtained appear in the following table as mg. per 100 grams of organ.

METHOD	RABBIT	DOG	CAT
Myers and Fine	518	367	449
Folin	510	441	496

Though the results for the rabbit agree in both cases closely, those for the dog obtained by Folin's method are higher. The individual estimations of Folin and Buckman vary considerably among each other, while Myers and Fine's values often show remarkable regularity. In the following table the authors have carried out a series of comparative analyses. In each instance the same material was employed for all methods.

Comparative creatine analyses of muscle.

(mg. per 100 gm.)

SPECIES	AUTHORS' EXTRACTION METHOD	FOLIN	BAUMANN
Dog	446	508	437
Dog	423	475	400
Dog (1)	482	507	420
Dog (2)	377	398	
Dog	454	492	
Dog	470	501	
Cat	579	581	532
Rabbit	576	583	553

As before, the results obtained by use of Folin's method are considerably higher for dog muscle. We are unable to verify the close correspondence reported by Baumann for his method, as compared to this author's determinations made in the same material by extraction.

[3] V. C. Myers and M. S. Fine: this *Journal*, xiv, p. 9, 1913.

[4] O. Folin and T. E. Buckman: *ibid.*, xvii, p. 483, 1914.

Further muscle experiments. (1) The muscle residue extracted free of creatine in analysis (1) of the table (authors' method) was treated precisely according to Folin's directions for the determination of creatine. Result: 23 mg. calculated for 100 gm. of fresh muscle. This corresponds almost exactly to the difference, 25 mg., between the analyses recorded in the table, according to the authors and to Folin.

(2) Dog muscle was coagulated by heating in water, and extracted with water, alcohol, and ether. As a control the residue was examined according to the authors' extraction method for creatine. Creatine = 0.9 mg. per 100 gm. of muscle. Hydrolysis with 25 per cent sulphuric acid for fourteen hours, and then subsequent examination for creatine was carried out on the extracted material. Colorimetric readings corresponding to 64.1 mg. creatine per 100 gm. of fresh muscle were found to be given by this material from which practically all creatine possible had previously been removed by extraction.

(3) To determine further the effect of heat and acid, 5 gm. taken from the same muscle used in analysis (2) of the table were hydrolyzed fourteen hours with three parts by weight of concentrated sulphuric acid and six parts of water, and thenceforward determined essentially according to Baumann. Result: 465 mg. per 100 gm. of fresh muscle.

(4) Rabbit muscle was hydrolyzed according to Folin, then shaken with ether, and the ether extract carefully separated and evaporated to dryness. The residue taken up with water gave a faint *atypical* Jaffé reaction. The color developed slowly, similar to the coloration given under the same circumstances by levulinic acid. Dog muscle, in like amount, was treated in precisely the same fashion. The ether residue dissolved in water gave a similar positive but atypical Jaffé reaction. The coloration was very deep. Weyl's reaction was negative. This ether-soluble substance is evidently not creatinine. Nor is it levulinic acid, as Weyl's reaction is positive for this acid.

From the above data we may conclude that the action of heat and acid on dog muscle leads to the production of products other than creatine, which are also the source of the higher results obtained by the use of the Folin method. These products are evidently not formed in sufficiently large amounts in rabbit muscle to interfere with the results in this case. The same probably holds true for cat muscle.

The creatine content of organs other than muscle.

For a comparative study of methods for the determination of creatine in other organs, the liver was made the especial object of examination.

With the employment of the extraction method the following results have been obtained per 100 gm. of liver: Beker, average of seven analyses, various species, 18.4 mg.;[5] Shaffer, dog, 15 mg.;[6] with the authors' method, dog, average of twelve analyses, chiefly fasting animals, 20 mg.; calf, 35 mg., average of two analyses. *Average result of fifteen determinations carried out according to Folin, chiefly fasting animals, was for the dog, 109 mg.*

The following determinations were made with the identical material in each case:

Comparative creatine analyses of organs.
(mg. per 100 gm.)

MATERIAL	EXTRACTION	HYDROLYSIS	
	Authors' method	Folin	With 5N H_2SO_4
Calf liver	45	95	
Dog liver	41	90	218
Dog liver	12	60	
Dog† liver	22	105	
Dog* liver	13		124
Dog liver	14	56	
Dog kidney	14	71	
Dog brain	112	173	

† Liver rich in glycogen.

The determinations made with the help of 5N sulphuric acid were carried out according to the method proposed by Baumann for muscle. Baumann has not recommended this procedure for liver. We have merely applied it to liver to obtain comparative results.

Additional experiments. (1) The *extracted* material obtained by employment of the authors' method from the determination starred (*) in the above table was treated precisely as the duplicate determination made with 5N sulphuric acid. Result: 137 mg. calculated for 100 gm. of fresh liver.

(2) Dog liver was coagulated and extracted with water and alcohol. The extracted liver was then examined for creatine with the following results. The results, calculated for 100 gm. of fresh material are, authors' method, 1.1 mg.; Folin, 63 mg.; hydrolysis fourteen hours with 33 per cent sulphuric acid, 214 mg.

[5] J. C. Beker: *Ztschr. f. physiol. Chem.*, lxxxvii, p. 21, 1913.

[6] P. A. Shaffer: this *Journal*, xviii, p. 525, 1914.

(3) Calf liver was prepared as above (2). A control creatine determination by the authors' method resulted negatively. Samples of this material were heated for periods of time varying from three to fourteen hours with acid as follows: normal hydrochloric, 5N (24.5 per cent) and 33 per cent sulphuric. Determinations then made essentially according to Baumann's technique gave colorimetric readings varying from 152 to 213 mg., calculated per 100 gm. of fresh liver.

The data presented above demonstrate that the action of mineral acid on liver leads to an increase in the colorimetric creatine values. In the case of the Folin method, as applied to liver, this increase may be 400 per cent higher than results obtainable by the employment of extraction. Higher results with the Folin method have also been obtained in organs other than liver and muscle. The conclusion is obvious. Either relatively large amounts of creatine, which cannot be removed by extraction, are set free by the action of heat and acid, or the effect of acid hydrolysis is to cause the production of other substances giving the Jaffé reaction. A definite decision of this question is of considerable importance (compare Introduction).

A search for creatine among the products of acid hydrolysis, with a view to the possible presence of other substances responding to the Jaffé reaction was, therefore, undertaken as follows:

Calf liver was carefully coagulated and extracted with water and alcohol as before. A control creatine determination by the authors' method in a sample of this material resulted negatively. An estimation according to Folin indicated the presence of 3.1 gm. of creatine in 1121 gm. of extracted liver. Other samples were boiled with three parts by weight of concentrated sulphuric acid and six parts of water, three hours and fourteen hours respectively, and then examined for creatine as described under muscle. In a like amount of material colorimetric readings denoted the presence of 11.1 gm. of creatine after three hours' hydrolysis, and 13.8 gm. after fourteen hours. The extracted liver (1121 gm.) was accordingly hydrolyzed with sulphuric acid fourteen hours at 110° precisely as was the sample. This length of time was chosen, as during it a maximal development of products yielding the Jaffé reaction took place. Creatinine is entirely unaffected by this treatment.[7] Creatine, if present, would be converted into creatinine.

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[7] Creatinine, contrary to the statements in the literature, is very stable in the presence of mineral acid, as the following experiment demonstrates: Creatinine, 0.104 gm., was boiled fourteen hours with 70 cc. H_2O and 30 cc. concentrated H_2SO_4. Creatinine recovered by analysis, 100 per cent. See also Baumann: *loc. cit.*

From the hydrolytic fluid so obtained the bases were precipitated by phosphotungstic acid in the usual manner. The chief amount of hydrolytic products giving the Jaffé reaction failed of precipitation here, and, therefore, could not be creatinine. For fear of loss of possible contained creatinine, the removal of ammonia was omitted. The phosphotungstic precipitate was decomposed with baryta solution saturated at 40°, working as expeditiously as possible. This procedure has been successfully employed by Kutscher in isolating creatinine from muscle extract, and evidently does not lead to a considerable loss of the creatinine by the action of barium hydroxide at the temperature and during the length of time employed. To the filtrate from the barium phosphotungstate, sulphuric acid was added to weak acid reaction. After the barium sulphate had been removed, the concentrated filtrate was treated with silver nitrate solution. The precipitate containing purines was removed by filtration, after standing twenty-four hours, and then additional silver nitrate solution was added in very slight excess. The histidine fraction was precipitated as silver salts by the careful addition of cold saturated baryta solution, filtered off, and washed with water containing barium hydroxide. The arginine and lysine fractions were separated from the histidine filtrate, and the silver salts of the three fractions were decomposed in the usual manner. The solutions of the bases were tested for the presence of creatinine. The histidine fraction gave a weak Jaffé reaction and Weyl reaction, to which tests the remaining fractions failed to respond.

From the histidine fraction the histidine and cytosine were separated by fractional precipitation with mercuric sulphate[8] and examined for the presence of creatinine with negative results. From the filtrate the mercury was removed by hydrogen sulphide, the sulphuric acid with baryta, and the neutral final solution evaporated to small bulk. In it the Jaffé and Weyl reactions were positive. A quantitative determination indicated the presence of 16.6 mg. of creatinine calculated as creatine. After acidulating and extracting with ether the solution failed to respond to Jaffé's reaction and Weyl's reaction. On evaporation to dryness a crystalline residue of about 0.1 gm. was obtained. This was not studied.

Under the conditions described above creatinine silver is precipitated in a solution of one part of creatinine to 3000 parts of water acidulated with sulphuric acid.[9] Precipitates do not form for several days even in strong creatinine solutions after the addition of mercuric sulphate in sulphuric acid.[10] Creatinine, if present, should therefore be found in the histidine fraction of bases, after removal of the histidine and cytosine. Here it was undoubtedly absent.

[8] A. Kossel and H. Steudel: *Ztschr. f. physiol. Chem.*, xxxviii, p. 49, 1903.

[9] Authors' observation (Janney).

[10] Authors' observation (Janney).

The ether extract, obtained as above, was evaporated to dryness, and the minute residue was dissolved in a little water. In this solution Jaffé's reaction was positive but atypical, as the color developed more slowly than in the case of creatinine. Weyl's reaction was positive but somewhat atypical. A typical Weyl reaction was given previously by the histidine fraction. Further examinations could not be attempted.

Acetyl-propionic (levulinic) acid has been prepared by heating with sulphuric acid nucleic acid derived from various organs.[11] It is also a derivative of lipoids, and is produced by heating practically any hexose with mineral acid. Acetyl-propionic acid is soluble in ether, not precipitated by phosphotungstic acid, and gives a strongly positive Jaffé and Weyl reaction. To the coloration produced by picric acid and alkali Folin has called attention. The full development of the color requires a longer time than in the case of creatinine, but it is more lasting. These properties coincide with those exhibited by the ether-soluble substance just described. This, we are therefore inclined to believe, represents a trace of acetyl-propionic acid, which failed of separation from the phosphotungstic acid precipitate through washing.

In the investigation just detailed all the operations were carried out with especial care in spite of the large amount of material it seemed advisable to employ. According to the colorimetric determinations, from 3.1 to 13.8 grams of creatinine calculated as creatine were present among the hydrolytic products. Of this no trace could be even *qualitatively* detected. Failure to find any evidence whatsoever of the presence of creatinine can scarcely be ascribed to the methods employed. On the other hand, the presence of another substance, probably acetyl-propionic acid, giving a very decided Jaffé reaction, could be established. Acetyl-propionic acid or similar products arising from liver hydrolysis are then the probable sources of the colorimetric findings ascribable to creatinine.

Fairly clear evidence, it is believed, has been presented above that the determination of creatine according to Folin cannot be recommended for the liver. This same conclusion can be drawn from the comparative analyses made in organs other than liver or muscle. As it has been shown for muscle that the Folin method, though exact

[11] P. A. Levene: *Ztschr. f. physiol. Chem.*, xliii, p. 199, 1904–05.

for some species, yields results in at least one other about 9 per cent too high, this method cannot be regarded as of general applicability to muscle determinations.

The Baumann muscle method, strangely enough, usually yields results considerably lower than the authors' extraction method, the accuracy of which has been rather rigorously controlled. We have found the technique of the Baumann procedure relatively inconvenient as well. From the data stated in this article the principle of hydrolysis with acid is to be avoided in the estimation of creatine in complex tissues.

The determination of creatine in muscle and other organs by extraction.

The accurate determination of creatine in organs has been found to be a task of some difficulty, especially in view of the very small amounts of this substance at times present. This is all the more true as no other adequate method has as yet been devised which could serve as a control for the Folin colorimetric procedure, the single one now applicable. For all determinations of creatine or its anhydride, including the method described in this paper, the possibility of error through the picric acid and alkali coloration being due in part to other substances than creatine, must be kept in mind.

The procedure described below is to be regarded essentially as a modification of that employed by Myers and Fine. Practically every step has been subjected to careful controls. Several sources of error, seeming hitherto to have escaped detection, have been discovered in this way. This procedure, in the authors' experience, has been found to possess advantages over previously described quantitative methods for the estimation of creatine in muscle and other organs. It has been in use in this laboratory for about one year.

Extraction of creatine from muscle and organs.

Among those reporting determinations of creatine in animal tissues by the employment of extraction, Riesser[12] and also

[12] O. Riesser: *Ztschr. f. physiol. Chem.*, lxxxvi, p. 415, 1913.

Myers and Fine[13] have described methods in full and definite detail. Although accurate determinations can be made by using these procedures, the employment of 45 or more grams of the organ to be examined and a correspondingly large volume of extract, renders their technique rather inconvenient.

It is, however, possible to overcome all serious objections to the determination of creatine by extraction if a small amount of the tissue is taken for examination, and the removal of the proteins be judiciously managed. By heating the finely divided organ at about 90° in water with the addition of about 15 drops of $\frac{N}{2}$ acetic acid, perfect coagulation and easy filtration may be attained. In all the following control experiments the technique employed was the same as given below under detailed description of the method.

Control of extraction method.

(mg. creatine per 100 gm.)

ORGAN	ORIGINALLY PRESENT	CREATINE ADDED	THEORETICAL	FOUND	ERROR
					per cent
Muscle	470	159	629	631	0.4
Brain	110	159	269	268	0.4
Liver	11	159	170	167	1.8
Kidney	14	159	173	173	0

It is evident that creatine can be quantitatively recovered from various organs by the authors' method.

As further controls, the extracted protein material from analyses of muscle and organs was *reextracted* according to the authors' technique a second and third time. These extracts were then further examined for the presence of creatine as in the method itself. In some of such extracts the coloration produced in the picric acid solution by the action of alkali was no deeper than that developing in distilled water, treated in like manner. In other extracts a minimal coloration corresponding at most to 1 mg. of creatine per 100 grams of organ was recorded. Though in muscle determinations the apparent error from this source would be negligible, in the case of organs containing minimal amounts

[13] Myers and Fine: this *Journal*, xvii, p. 65, 1914.

of creatine this would not hold true. As no difference in the intensity of this faint coloration could be found, whether in organs or muscles, though in the latter case thirty to forty times more creatine was originally present, the cause of this coloration can hardly be attributed to creatine not removed by the original extraction and washing as carried out in the method detailed below.

Although the help of a protein precipitant is not a necessity, we have found it convenient to add aluminium hydroxide, as then less care need be given to the coagulation of the protein, the use of acid can be omitted; and filtration is somewhat more rapid. That creatine is not absorbed, nor its extraction from the tissues retarded under these circumstances, the following control experiments demonstrate. The amount of aluminium hydroxide added was 1.5 grams or more.

The effect of addition of aluminium hydroxide on the determination of creatine.
(mg. per 100 gm.)

MATERIAL	ANALYSIS	CONTROL ANALYSIS
	(Addition of $Al(OH)_3$*)*	*(Heat and acid)*
Rabbit muscle	578	576
Dog muscle	444	446
Dog muscle	428	423
Dog muscle	454	453
Cat muscle	581	579
Dog liver	24	24
Dog brain	124	124

The conversion of creatine into creatinine in complex organ extracts is best accomplished on the water bath and in dilute solution. Even the water-clear protein-free extracts may contain substances, possibly carbohydrates, which produce the creatinine coloration after heating with acid.

In the hope that the *autoclave* could safely be employed after complete separation of the proteins, in various instances the conversion was carried out according to Folin's directions. The results were invariably higher in the case of organs other than muscle, than those of control analyses made according to the authors' procedure, with use of the water bath. Usually the dif-

ference observed amounted only to a few milligrams, but occasionally the error so introduced is of a serious nature. That these higher values in the case of the autoclave are not ascribable to failure of complete conversion in the case of the procedure described in this paper, we have also determined by special experiments with pure creatine solutions and organ extracts. The protocols of these and following control experiments are omitted for the sake of brevity.

Glucose and urea in concentrations up to 1 per cent in the organ extract alone or together have no influence on the determination of creatine according to the proposed method. In these control experiments, also in determinations made on livers rich in glycogen, a new development of color begins about half an hour after the addition of the alkali to the picric acid solution. It is, therefore, essential to make the colorimetric observations promptly.

Owing to the smaller amounts of creatine found in organs other than muscle, we regard it advisable to concentrate the extracts, though more time is required for this. In this case 0.5 normal sulphuric acid has been found in another series of control experiments, sufficient to convert all the creatine present. The determination of creatinine, according to Folin,[14] may be employed when separate determinations of both creatine and its anhydride are required in organs. Whether the proposed method is applicable to organs gorged with blood (perfused liver) has not as yet been definitely determined.

The determination of creatine in muscle. *(Method in detail.)*

The fresh muscle is quickly freed from visible connective tissue, cut fine, preferably by the meat grinder, and thoroughly mixed. From a sample in a weighing glass provided with a ground glass lid, about 5 grams are weighed by difference into a beaker glass, stirred up in 50 cc. of water, and heated to the point of boiling. The flame is then removed. 10 cc. of a 15 per cent suspension of powdered aluminium hydroxide are stirred through the con-

[14] Folin: *loc. cit.*

tents of the beaker, which are filtered hot through a fairly small Buchner funnel into a previously warmed flask. Extraction is carried out with 150 cc. of hot water in six portions, care being taken to fill the funnel completely and suck without undue pressure, nearly to dryness each time.

The water-clear filtrate is transferred quantitatively to a flask graduated at 300 cc.,[15] and the filter flask washed with 55 cc. of 25 per cent sulphuric acid, followed by water until the volumetric flask contains about 280 cc. of fluid. The acid concentration is then approximately normal. Conversion is effected by three hours' immersion in a boiling water bath. After cooling, the contents are diluted to the mark, and thoroughly mixed. Correspondingly smaller amounts of muscle, aluminium hydroxide, volume of extract and sulphuric acid may safely be employed, provided the weighing of the material is carefully done.

Folin's colorimetric procedure is followed from this point. It is repeated for convenience. The acidity of 10 cc. of the converted solution is determined by titration with a 10 per cent solution of sodium hydroxide, phenolphthalein being used as indicator. 10 cc. of the unknown and of a standard solution, containing creatinine equivalent to 1 mg. of creatine in 10 cc. are pipetted respectively into 100 cc. volumetric flasks and to each 20 cc. of saturated picric acid solution are added. Sufficient 10 per cent sodium hydroxide solution to give 1.5 cc. over and above that required for neutralization of the sulphuric acid is allowed to run into the unknown solution from a burette. To the standard solution 1.5 cc. of the alkaline solution are added in like manner. After ten minutes the solutions are diluted to the mark and read, the standard being set at 10 or 15 mm.

Method in detail for organs other than muscle.

The technique is precisely the same in this case as in muscle determinations up to and including extraction of the creatine,[16]

[15] Small Jena flat bottom flasks graduated to 300 cc. are conveniently used here.

[16] Aluminium hydroxide, however, is best omitted in the determination in brain, where heat and acid are preferable (see above).

except that less than 5 grams of material should be employed only under necessity. 8 cc. of 25 per cent sulphuric acid are added to the creatine extract, which is transferred to a porcelain dish, the filter flask being washed twice with a little water. The contents of the dish are evaporated on a boiling water bath down to a volume of about 75 cc. The acid concentration is now about 0.5 normal. The dish is then covered with a large watch crystal to retard further evaporation, and the conversion completed by a two hours' continuance of the heating on the water bath. The cooled solution is quantitatively transferred to a 100 cc. volumetric flask, diluted to the mark, and mixed.

The colorimetric determination is made as in muscle. Standards which may be used contain creatinine equivalent to 0.5, 0.25, 0.125, and 0.0625 mg. of creatine in 10 cc. solution. In working with organs containing but traces of creatine, the use of a volumetric flask at this step can best be entirely dispensed with. 10 cc. of the unknown are pipetted into a small glass, 20 cc. of picric acid solution added in like manner, and from a burette 1.5 cc. of 10 per cent sodium hydroxide solution in addition to the amount necessary to neutralize 10 cc. of the unknown. To 10 cc. of the standard are given, in like manner, 20 cc. of the picric acid, and 1.5 cc. of the alkali solution. In addition, sufficient distilled water is allowed to run in from a burette to make the volume equal that of the unknown. In all cases the standard in the colorimeter may be set at 20 mm.

Organ analyses.

The results of organ analyses are appended in the following table. They substantially agree with those previously obtained by Beker,[17] who has also called attention to the relatively large amounts of creatine in brain and testes. Fasting, feeding, or phlorhizin injections exert no obvious influence on the amount of creatine found in the liver.

[17] Beker: *loc. cit.*

Creatine content of organs.
(mg. per 100 gm.)

SPECIES	ORGAN	CREATINE
Dog	Brain	124
"	"	119
"	"	112
"	"	110
"	Pancreas	18
"	Kidney	27
"	"	14
"	Spleen	30
"	Testes	181
"	Liver*	20
Calf	"	45
"	"	26

*Average of twelve analyses (12 to 45 mg.).

Concerning the relation of creatine to protein.

Creatine, as has been shown above, can be entirely removed from the liver by hot water. From such extracted liver protein, boiling with strong mineral acid under the conditions usually adopted for protein hydrolysis, fails to yield additional amounts of this substance. That creatine is to be regarded as an integral part of liver protein or of possible other higher complexes broken down by acid is therefore improbable. Apparently the only positive evidence for accepting that creatine represents an integral part of organ protein seems to have been the higher colorimetric readings obtained in analyses involving hydrolysis of the tissues with acid. These results, as seen above, are due to substances other than creatine. The failure of discovery of this substance among the products found in hydrolyzing muscle proteins (Osborne and coworkers) is additional evidence against the existence of creatine in firm combination in such protein substances.

The view that creatine is *loosely* combined with living protoplasm has been advanced by Urano[18] from Hofmeister's laboratory. The value of the experimental data of Urano's work may, however, aside from analytical inadequacies, be fairly questioned. That greater amounts of creatine in his experiments were given

off to media by hashed or preserved muscle, may be due to direct mechanical causes, or to an increased permeability of the cell membranes for creatine.

Folin,[19] who advocates Urano's hypothesis, has demonstrated that creatine present in lower concentration in blood is absorbed by muscle, in which *post mortem* this substance is found to be present in much higher concentration. The conclusion reached is that virtually no creatine can be present free in living muscle, for otherwise such absorption could not occur.

Certain objections to acceptance of this view, that creatine is present loosely combined in protoplasm, may, however, be raised. Examples are known where absorption of substances from low concentration in body fluids into areas of higher concentration can occur without chemical union. Fat has been found to be present in a concentration of 3 to 5 per cent at most in lymph, from which it can be absorbed by tissues already laden with fat. It seems also difficult to understand why creatine, which may be regarded as a glycine derivative, should be capable of being split off by the mere death of organs, when all other amino-acids require boiling with mineral acid to set them free. Moreover, if we accept that creatine is combined in the living cell substance, possibly the remaining muscle extractives, including urea, might have to be included in the concept, protoplasm. This seems scarcely acceptable. There seems, at least, no very definite evidence as yet recorded that creatine exists even in a loose state of combination in organs.

The acceptance of actual chemical union does not seem necessary to explain the close connection with protein metabolism so clearly established for creatine. This substance may not unlikely be synthesized from the products of protein breakdown, possibly to serve normally as a highly nitrogenous reserve product, in a manner somewhat analogous to liver glycogen in carbohydrate metabolism.

SUMMARY.

Creatine and creatinine are probably not to be regarded as existing in firm combination in liver and muscle; as acid hydrolysis

[18] F. Urano: *Beitr. z. chem. Phys. u. Path.*, ix, p. 104, 1907.
[19] O. Folin and W. Denis: this *Journal*, xvii, p. 493, 1914.

of such organs, previously freed of these substances by extraction, fails to yield additional creatine or its anhydride.

To previously described methods for the determination of creatine in muscle or other organs various criticisms can be offered. Folin's procedure for the estimation of creatine in muscle is of limited applicability. For organs other than muscle it cannot be recommended.

Improved methods for the determination of creatine in muscle and other organs are described.

THE METABOLISM OF CREATINE AND CREATININE.

TENTH PAPER.

THE RELATIONSHIP BETWEEN CREATINE AND CREATININE IN AUTOLYZING TISSUE.

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Despite active investigation, evidence has not been forthcoming to show clearly the relationship between creatine and creatinine in the animal body. Still less has been determined with regard to the precursors of these substances.

In 1907 Benedict and one of us[1] called attention to the fact that when carefully preserved urines were allowed to stand, the creatinine was slowly changed to creatine. We expressed uncertainty as to whether this change was chemical or enzymatic. Several months later there appeared the first of a series of papers by Gottlieb and his coworkers[2] dealing with the formation and destruction of both creatine and creatinine. By an elaborate investigation of the behavior of these compounds during autolysis, Gottlieb and Stangassinger[3] have concluded that they undergo a series of enzymatic transformations in the body, the more important of which are: (1) creatine is formed in autolysis of various tissues; (2) preformed or added creatine can be converted by enzymatic means into creatinine during autolysis; and (3) both of these compounds may be destroyed by appropriate enzymes, "creatase" and "creatinase." This was disputed by Mellanby,[4] but after a repetition of the above work van Hoogenhuyze and Verploegh,[5] and Rothmann[6] maintained the essential importance of endo-

[1] F. G. Benedict and V. C. Myers: *Am. Jour. Physiol.*, xviii, p. 404, 1907.

[2] R. Gottlieb and R. Stangassinger: *Ztschr. f. physiol. Chem.*, lii, p. 1, 1907.

[3] Gottlieb and Stangassinger: *ibid.*, lii, p. 1, 1907; lv, p. 322, 1908. Stangassinger: *ibid.*, lv, p. 295, 1908. A. Rothmann: *ibid.*, lvii, p. 131, 1908.

[4] E. Mellanby: *Jour. Physiol.*, xxxvi, p. 463, 1907–08.

[5] J. C. van Hoogenhuyze and H. Verploegh: *Ztschr. f. physiol. Chem.*, lvii, p. 206, 1908.

[6] Rothmann: *loc. cit.*

enzymes in the metabolism of the compounds under discussion and experiments have further been reported by Rowe,[7] Inouye,[8] and Palladin and Wallenburger,[9] which, in general, support this view.

Although many of the essential points brought out by the experiments of Gottlieb and Stangassinger have been confirmed, their work has not been generally accepted. In discussing this subject Folin and Denis[10] state: "The hypothetical transformation of creatinine into creatine in the muscles, as suggested by Mellanby, or by means of ferments, as claimed by Gottlieb and Stangassinger, is not founded on convincing experiments and is certainly not supported by the determinations recorded above" (experiments on the living cat). In a further discussion of the work of the Heidelberg investigators they write: "To be sure van Hoogenhuyze and Verploegh have verified their results but Mellanby on the other hand was unable to do so." These statements typify the scepticism with which this subject has been viewed by workers in this field.

With regard to the work of Gottlieb and Stangassinger, it may be mentioned that their results for preformed creatinine were obtained with a method requiring evaporation by heat (barium carbonate being added to keep the reaction neutral). Although their data appear to lend support to their method, the use of heat is open to criticism. An inspection of Mellanby's muscle autolysis experiments shows that he was unable to detect the presence of creatinine in experiments in which it must have been present in appreciable amounts. In view of this fact his severe criticisms of the work of Gottlieb and Stangassinger are without significance.

The experiments reported in the present paper were carried out with the object of clarifying some of the disputed points.

Methods employed.

The methods employed for the determination of the creatine and creatinine in the tissues, except as noted below, were those described in an earlier communication.[11] In the case of autolyzing muscle the methods were found to give most satisfactory

[7] A. H. Rowe: *Am. Jour. Physiol.*, xxxi, p. 169, 1912–13.

[8] K. Inouye: *Ztschr. f. physiol. Chem.*, lxxxi, p. 71, 1912.

[9] A. Palladin and L. Wallenburger: *Compt. rend. Soc. de biol.*, lxxviii, p. 111, 1915.

[10] O. Folin and W. Denis: this *Journal*, xii, p. 152, 1912.

[11] V. C. Myers and M. S. Fine: *ibid.*, xvii, p. 65, 1914.

results. In some of our later experiments requiring the estimation of very small amounts of creatine and creatinine the recent methods of Folin[12] have been used (data in Tables VI, X, XI, XII, and XIII).

EXPERIMENTAL PART.

Before considering the changes in the creatinine and creatine content of any tissues undergoing autolysis, it appeared essential to ascertain what changes, if any, might take place in pure solutions of these substances. In agreement with Gottlieb and Stangassinger,[13] it was found that creatine in pure solution was slowly transformed to creatinine (Table I), the rate at 36°C. being

TABLE I.

Formation of creatinine in a solution of pure creatine (0.2 per cent).

SAMPLE	PROPORTION OF CREATINE TRANSFORMED TO CREATININE	PERIOD OF AUTOLYSIS	TEMPERATURE. REMARKS
	per cent	*weeks*	
1	0.24	1	0°C. Almost no change.
2	3.1	1	36°C.
3	7.2	2	36°C.
4	8.6	3	36°C.
5	12.7	4	36°C.

slightly less than 0.5 per cent per day for the first few weeks. A second set of experiments (Table II) in which creatinine was employed showed that it was transformed to creatine in a similar manner; at the end of eleven months an equilibrium had been reached in both cases. This suggests that the phenomenon is a reversible reaction in which an equilibrium is gradually reached between the two substances. Evidently creatine and creatinine may be changed each to the other, even in pure solution, although as will be noted in experiments to follow, the rate is much slower than that found in the autolysis of muscle and other tissue. Determinations of total creatinine show a loss of about 10 per cent in both cases. If the creatine and creatinine are decomposed

[12] O. Folin: *ibid.*, xvii, p. 475, 1914.

[13] Gottlieb and Stangassinger: *Ztschr. f. physiol. Chem.*, lii, p. 1, 1907.

it is quite natural to expect that this would be in part through a formation of sarcosine and urea. Controlled estimations of urea with the aid of soy bean urease and Nessler's solution gave about 0.5 mg. of urea nitrogen in each case, which would account for about 2 per cent of the creatine and creatinine lost. This amount is, we believe, slightly beyond the limits of experimental error.

TABLE II.

Changes in pure solutions of creatine and creatinine.

DATE 1914–15	PERIOD OF INCUBATION AT 36°C.	TOTAL CREATININE	CREATININE	CREATINE IN TERMS OF CREATININE
Solution of pure creatinine, 100 mg. to 100 cc.				
	days	*per cent*	*per cent*	*per cent*
June 26	0	100	100	0
July 9	13		86	
Aug. 18	53		74	
Aug. 24	59	90	65	35
Oct. 17	113		49	
Feb. 17	246	90	42	48
May 19	337	90	40	50
Solution of pure creatine, 100 mg. to 100 cc., in terms of creatinine.				
June 26	0	100	0	100
July 9	13		9	
Aug. 18	53		29	
Aug. 24	59	90	30	60
Oct. 17	113		37	
Feb. 17	246	90	39	51
May 19	337	90	40	50

Table III gives the changes in the creatinine (and creatine) content of urines refrigerated for one year. The two sets of experiments show comparable results. The last specimen in each case was allowed to stand in the room some time before being refrigerated. Judging from the results with pure solutions, an equilibrium point had nearly been reached in the last two specimens. The amount of creatinine lost was the same as with the pure solutions.

As has already been pointed out, the creatinine (as well as the creatine) concentration of muscle tissue is much higher than that

of any other body tissue,[14] which strongly points to the muscle as the chief site for the formation of creatinine. A study of autolyzing muscle tissue might therefore be expected to throw light upon the relationship between creatine and creatinine. For this reason, as well as for the fact that the work of Gottlieb and his coworkers was conducted largely on other tissue, our attention

TABLE III.

Changes in the creatinine of urines refrigerated for one year.

SOURCE OF URINE		ORIGINAL CONTENT OF CREATININE	TOTAL CREATININE FOUND	PREFORMED CREATININE	CREATINE
		Nov., 1913	Nov., 1914		
		gm.	*gm.*	*gm.*	*gm.*
V. C. M.	3	1.35	1.21	1.04	0.20
	4	1.35	1.21	0.92	0.34
	5	1.35	1.23	1.01	0.26
	6*	1.35	1.23	0.60	0.73
Averages			1.22	0.89	0.38
M. S. F.	3	1.29	1.11	0.85	0.30
	4	1.29	1.11	0.85	0.34
	5	1.29	1.14	0.81	0.38
	6*	1.29	1.14	0.50	0.74
Averages			1.13	0.75	0.44

	V. C. M. *per cent*	M.S.F. *per cent*
Creatinine unchanged	66	68
Creatinine changed to creatine	24	20
Creatinine lost	10	12

* Urine No. 6 in both experiments was allowed to stand at room temperature for a considerable period before being refrigerated. Toluene was used as preservative in all cases.

has been directed chiefly to muscle tissue, although some consideration has been given to blood and liver.

As will be noted in Table IV, when the finely ground muscle tissue of the rabbit is allowed to autolyze in the presence of a little more than an equal volume of water with toluene as a preservative, the creatinine increases at a very constant rate at the expense of the creatine. The rate of the reaction gradually

[14] Myers and Fine: this *Journal*, xxi, p. 387, 1915; see also *Proc. Soc. Exper. Biol. and Med.*, xi, p. 15, 1913.

slows up until an equilibrium point is reached. The rate of transformation here is more than three times as great as in pure solution. Should this phenomenon prove to be other than a post mortem change, it would be quite sufficient to explain not only the *amount* of the creatinine elimination, but also the *constancy* in

TABLE IV.

Influence of time.

MUSCLE SAMPLES	PERIOD OF AUTOLYSIS AT 36° C.	CREATININE FORMED		TOTAL CREATININE AS CREATINE
		Per 100 gm. muscle	Per 100 gm. muscle per day	
Rabbit 67. June, 1913.				
	days	*mg.*	*mg.*	
1, 2	0	8.8, 11.6		0.508
3, 4	4	51.3, 51.3	10	0.508
5, 6	9	82.7, 81.0	8	0.508
7, 8	13	100.0, 100.0	7	0.508
9, 10	18	128.6, 117.5	6	0.508
Rabbit 77. Sept., 1913.				
1	0	6.8		0.553
3	2	20.2	6.7	0.553
4	4	36.6	7.4	0.553
5	6	50.6	7.3	0.553
6	8	62.5	7.0	0.553
7	10	72.3	6.7	0.553

its excretion. As will be noted in this table, there appears to be no destruction of either creatine or creatinine in rabbit muscle under the conditions of our experiments.

As is well known, the excretion of creatinine is increased in fever. That this is quite in proportion to the rise in body temperature and probably due *entirely* to the hyperthermia has al-

ready been pointed out.[15] In this connection it is of considerable interest to learn the influence of comparatively small changes in temperature on the rate of autolysis. It will be noted in Table V that autolysis at 36°C. produced 6 mg. of creatinine per 100 grams of muscle per day, while at 39°C., 9 mg. were produced, and at 44°C., 13 mg., which is entirely in harmony with our earlier observations *in vivo*. The marked similarity of the results obtained under the two conditions emphasizes the probable importance of the factors present in the experiments here reported. No explanation can be offered for the high figures for total creatinine found in muscle samples Nos. 5 and 6.

TABLE V.

Influence of temperature.

MUSCLE SAMPLES RABBITS 78 AND 79 MIXED NOV., 1913	LENGTH OF AUTOLYSIS	CREATININE INCREASE		TOTAL CREATININE AS CREATINE	TEMPERATURE OF AUTOLYSIS. REMARKS
		Per 100 gm. muscle	Per 100 gm. muscle per day		
	days	*mg.*	*mg.*	*per cent*	
1	0	6.8		0.495	Fresh muscle.
2	7	19.3	3	0.495	20°C.
3	7	44.4	6	0.501	36°C.
4	7	63.3	9	0.501	39°C.
5	7	89.0	13	0.561	44°C.
6	7	165.3	23	0.570	36°C. 2 cc. concentrated HCl added.
7	15 hrs.	207.7	200	0.527	15 hrs. at 85°C.
8	7	213.1		0.501	15 hrs. at 85°C., 6 days at 36°C.

It seemed important to learn whether either pure creatine or creatinine, when added to autolyzing muscle tissue, would experience the same fate as the creatine originally present. That creatinine when added to autolyzing muscle will inhibit the transformation of creatine to creatinine, or if added in sufficient quantity, cause the reaction to proceed in the opposite direction is evident from the first portion of Table VI. By the addition of 200 mg. of creatinine to Sample 4 containing 45 mg. of creatine (in terms of creatinine), the amount of creatine calculated as

[15] V. C. Myers and G. O. Volovic: this *Journal*, xiv, p. 489, 1913.

TABLE VI.

Influence of the addition of creatinine.

Muscle samples rabbits 80-81 mixed Nov., 1913	Creatine in 10 gm. muscle as creatinine	Creatinine added	Total creatinine	Total creatinine after 20 days' autolysis	Loss in total creatinine	Preformed creatinine at end of autolysis	Loss in performed creatinine	Creatine at end of autolysis as creatinine	Creatine at end of autolysis
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	45	50	95	95	0	49	1	46	53
2	45	100	145	138	8	86	14	52	60
3	45	150	195	182	13	117	33	65	75
4	45	200	245	230	15	159	41	71	82

Influence of the addition of creatine.

Muscle samples rabbits 80-81 mixed Nov., 1913	Creatine in 20 gm. muscle as creatinine	Creatine added as creatinine	Total creatine as creatinine	Total creatinine after 20 days' autolysis	Difference	Preformed creatinine at end of autolysis	Creatine converted to creatinine	Creatine unchanged as creatinine
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*mg.*
5	90	85	175	184	+ 9	44	23.9	140
6	90	170	260	271	+11	61	22.4	211
7	90	255	345	342	− 3	81	23.7	261
8	90	340	430	425	− 5	101	23.8	323

TABLE VII.

Influence of acetic acid and neutral phosphate mixture.

Sample	Period of autolysis at 36° C.	Creatinine increase calculated per 100 gm.		
		Muscle plus water	Muscle plus 0.02 N acetic acid	Muscle plus 1 per cent phosphate mixture
	days	*mg.*	*mg.*	*mg.*
1	3	21	67	20
2	6	34	126	42
3	9	60	185	80
4	12	70	200	88
5	21	98	232	109
6	28	111	253	114

creatinine was raised to 71 mg. at the end of twenty days. That added creatine experiences the same fate as the creatine originally present in the muscle tissue, is evident from the fact that there was essentially the same rate of transformation, whether plain muscle, muscle plus 85 mg. of creatine, or muscle plus 340 mg. of creatine was under observation.

As may be seen from Table VII, the velocity of the transformation of creatine to creatinine is increased by weak acid but not materially affected by Henderson's neutral phosphate mixture.

TABLE VIII.

Autolyses with cat muscle.

MUSCLE SAMPLES CAT 17 NOV., 1913	PERIOD OF AUTOLYSIS	CREATININE INCREASE Per 100 gm. muscle	CREATININE INCREASE Per 100 gm. muscle per day	TOTAL CREATININE AS CREATINE	TEMPERATURE OF AUTOLYSIS. REMARKS
	days	*mg.*	*mg.*	*per cent*	
3	0	4.8		0.482	Fresh muscle.
14	4	18.0	3	0.482	20°C.
15	8	23.1	2	0.482	20°C.
4	4	38.4	8	0.482	36°C.
5	8	62.3	7	0.482	36°C.
6	12	73.6	6	0.482	36°C.
7	16	100.0	6	0.482	36°C.
8	20	108.0	5	0.482	36°C.
9	28	119.1	4	0.482	36°C.
10*	56	143.6	3	0.482	36°C.
11*	84	186.0	2	0.482	36°C.
12	4	47.7	11	0.482	39°C.
13	8	95.3	11	0.482	39°C.
16	4	18.0	3	0	36°C. No toluene.
17	28	228.0		0.488	0.11 gm. creatine added to 25 gm. muscle.

* Bacteriological examinations gave negative results.

The autolysis experiments carried out with cat muscle are comparable in every respect with those reported for the rabbit (Tables IV and V); *i.e.*, there is a uniform rate in transformation of creatine to creatinine, which is increased with a rise in temperature, but is gradually retarded as the reaction approaches an equilibrium point. There is likewise no loss in total creatinine, indicating no destruction of either substance.

TABLE IX.

Autolyses with dog muscle.

MUSCLE SAMPLES DOG 6 NOV., 1913	PERIOD OF AUTOLYSIS AT 36° C.	CREATININE INCREASE		TOTAL CREATININE AS CREATINE
		Per 100 gm. muscle	Per 100 gm. muscle per day	
	days	*mg.*	*mg.*	*per cent*
1	0	5.0		0.418*
3	4	21.3	4	0.404
4	8	31.7	3	0.376
5	12	41.3	3	0.376
6	16	33.2	2	0.376
7	21	46.6	2	0.358
8	28	42.6	2	0.348
9	35	33.8	1	0.358

* Moisture content of muscle, 73.5 per cent; nitrogen, 3.37 per cent. Creatine calculated for 76 per cent moisture, 0.378 per cent.

TABLE X.

Creatinine and creatine content of the tissue of the fowl.

ANIMAL	TISSUE	CREATININE	CREATINE	SUGAR	REMARKS
		mg. to 100		*per cent*	
Hen 1	Blood	1.3	5.2	0.33	Creatinine determinations completed 1 hr. after death.
	Leg muscle	1.6		0.28	
	Breast muscle	5.3	483.	0.39	
	Liver	1.6	15.	0.98	
Hen 2	Blood	1.2	6.6	0.26	Creatinine determinations completed ½ hr. after death.
	Leg muscle	1.9	442.	0.28	
	Breast muscle	5.0	452.	0.42	
	Liver	1.2	11.5	1.84	
Hen 3	Blood	0.5	6.5	0.18	Creatinine was determined about 3 hrs. after blood was taken.
Hen 4		0.4	4.2	0.17	
Hen 5		0.4	5.3	0.17	
Hen 6		0.5	5.7	0.22	
Hen 7		0.6	5.5	0.24	
Hen 8		0.6	5.8	0.19	
Duck 1	Blood	0.67	5.4	0.13	Creatinine was determined about 2 hrs. after blood was taken.
Duck 2		0.83	5.9	0.14	
Duck 3		0.88	5.2	0.14	
Duck 4		0.60	5.1	0.14	

The results obtained in the autolysis of dog muscle are rather different from those obtained in the case of the rabbit and the cat. In the early stages of the autolysis there was a change of creatine to creatinine quite comparable to that previously obtained, but after a certain point had been reached there was no further increase in the creatinine of the present series, and there likewise appeared to be some loss in total creatinine. The cause of the difference between these and previous experiments is not clear. We do not believe that it was due to bacterial factors, since all bacteriological examinations, kindly made for us in these and other experiments by Dr. R. M. Taylor, were negative.

TABLE XI.

Autolysis of fowl muscle.

PERIOD OF AUTOLYSIS	CREATININE INCREASE PER 100 GM. MUSCLE			REMARKS
	Breast muscle Hen 1	Breast muscle Hen 2	Leg muscle Hen 2	
days	*mg.*	*mg.*	*mg.*	
0	5.3	5.0	1.9	
4	25.0	22.7		
7	38.8	38.7	35.7	Total creatinine of breast muscle unchanged.
11	65.0	63.3		
14	78.0	83.3		Total creatinine of breast muscle unchanged.
18	88.2	100.0		

It is possible that conditions are somewhat different in the dog. If this be true it may in part explain some of the rather peculiar results obtained by Gottlieb and Stangassinger, since most of their experiments were carried out on tissue from this animal.

The data which have been reported dealing with the metabolism of creatine and creatinine in the fowl indicate that it differs considerably from the metabolism of these substances in mammals. Paton[16] has claimed that creatine and not creatinine is

[16] D. N. Paton: *Jour. Physiol.*, xxxix, p. 485, 1909–10. D. N. Paton and W. C. Mackie: *ibid.*, xlv, p. 115, 1912–13.

found in the urine of birds, while Folin and Denis[17] have reported the creatinine content of the blood of the fowl to be 0.1 ± mg. per 100 cc.

It seemed important, therefore, to examine the influence of autolysis upon the creatine of the muscle in the fowl as well as the creatine and creatinine content of fresh tissue. From the data in Table X, the creatinine content of the blood of fowls does appear to be slightly lower than that of mammalian blood, although we have failed to find any figures approximating those reported by Folin and Denis. On account of the question of a

TABLE XII.

Autolysis of mixed human blood.

(Figures represent mg. to 100 cc.)

	Period of autolysis at 36°C.	A Nothing added			B Creatinine added 10 mg.			C Creatine added 11.6 mg.		
		Total creatinine	Preformed creatinine	Creatine	Total creatinine	Preformed creatinine	Creatine	Total creatinine	Preformed creatinine	Creatine
	days									
Heated	0	7.1	2.6	5.2						
	4	7.1	3.3	4.4	16.3	12.1	4.9	16.4	3.8	14.6
	10	7.1	2.0	5.9	16.7	10.0	7.8	17.1	3.8	15.4
Not heated	0	6.2	1.9	5.0	15.9	11.6	5.0	15.9	2.2	15.9
	4	8.5	4.5	4.6	18.5	12.8	6.6	17.9	4.4	15.7
	10	9.7	4.3	6.3	19.0	11.8	8.3	20.4	5.0	17.9

possible interference of sugar with the estimation of creatinine, sugar estimations were made. The results were negative in this respect, as may be observed from the table.

An inspection of the data presented in Table XI shows that creatine is transformed to creatinine in the autolyzing muscle of the hen in a manner directly comparable to that noted in the cat and rabbit. Since the excretion of the hen contains creatine instead of creatinine,[18] the above findings lend some support to the argument advanced by Folin and Denis that the

[17] Folin and Denis: this *Journal*, xvii, p. 491, 1914.

[18] Folin and Denis: *ibid.*, xvii, p. 501, 1914.

changes encountered in autolysis are of a *post mortem* nature. The excretion of creatine by the fowl is a topic in need of further investigation.[19]

A number of autolysis experiments has been carried out with tissue other than muscle, but only two sets will be reported. The first series of autolyses was conducted with mixed human blood, 5 cc. being diluted with 15 cc. of 0.9 per cent sodium chloride solution, and 2 cc. of toluene added as preservative. In the second set 2.5 gram portions of finely ground rabbit liver were diluted with 10 cc. of the saline and 2 cc. of toluene.

TABLE XIII.

Autolysis of rabbit liver.

(Figures represent mg. per 100 gm.)

Period of autolysis at 36° C.		A Nothing added			B Creatinine added 20.0 mg. per 100 gm.			C Creatine added 23.2 mg. per 100 gm.		
		Total creatinine	Preformed creatinine	Creatine	Total creatinine	Preformed creatinine	Creatine	Total creatinine	Preformed creatinine	Creatine
	days									
Heated	0	36.5	3.5	38.3						
	5	30.8	9.5	24.7				46.0	10.4	41.3 (18.1)
	10	22.5	10.9	13.9	44.4	24.7 (4.7)	22.9	45.0	12.1	38.2 (15.0)
Not heated	0	21.4	3.0	21.3	45.4	23.3 (3.3)	25.6	45.5	3.8	46.2 (23.0)
	5	40.0	9.1	35.9				58.0	10.2	55.5 (32.3)
	10	39.2	8.9	39.2	56.4	19.6 (−.4)	42.7	56.4	9.5	54.4 (31.2)

Folin's new methods[20] for the estimation of the creatine and creatinine (in blood) were employed in both cases. The material was made up to some definite volume, 25 or 50 cc., with distilled water, treated with sufficient dry picric acid to remove the proteins and saturate the solution, centrifuged, and filtered. For the creatinine estimations 10 cc. portions were treated with 0.5 cc. of 10 per cent sodium hydroxide and compared in the Duboscq colorimeter with standard solutions of creatinine (0.2, 0.5, and 1.0 mg. to 100 cc.) in saturated picric acid similarly treated. For the creatine 5 cc. portions were autoclaved in the usual way, diluted to

[19] In two cases we have determined that the preformed creatinine of the hen's excreta represents only 10 per cent of the total creatinine, supporting Paton's findings with regard to the excretion of creatine.

[20] Folin: this *Journal*, xvii, p. 475, 1914.

10 cc. with saturated picric acid, and further manipulation was carried out in the same way as for the creatinine.

An inspection of the control determinations in Table XII appears to support the general accuracy of Folin's method. The figures for the preformed creatinine in A and C show a progressive increase in the samples not heated, though in the heated controls there was essentially no change. In all the unheated specimens there was an increase in the creatine. This was most noticeable in B, where creatinine had been added, and inhibited any transformation of creatine to creatinine. In general it may be said the experiments on the blood support the preceding experiments, and in addition indicate an increase in the determinable amount of creatine.

The experiments in Table XIII are scarcely as easy of explanation as those reported in Table XII. Apparently a variety of factors is at work, and we obtain here results similar to those observed in many of the experiments of Gottlieb and Stangassinger. An inspection of the table, however, does appear to disclose the same increase in creatinine and creatine as noted in the experiments with blood.

DISCUSSION AND SUMMARY.

Our present study deals primarily with the relation between creatine and creatinine in pure solutions and in autolyzing tissues under various conditions. Such studies can at best constitute only indirect evidence as to the processes taking place in the body. Despite the indirect nature of the evidence, however, the results tend so persistently in one direction, that we have ventured to formulate a working hypothesis as to the metabolic relationship between creatine and creatinine.

From the data presented it is evident that creatine is transformed to creatinine, and creatinine to creatine, even in pure solution, at a very constant rate which represents about 0.5 per cent per day at body temperature. This should be borne in mind in any study of the relation between these two substances.

In muscle tissue autolyzing at body temperature the rate of the transformation of creatine to creatinine is sufficiently rapid (more than three times that in pure solution) to be almost directly com-

parable with the rate of creatinine formation in the body. Since the creatinine is removed by the kidney almost as rapidly as it is formed, this (supposed) transformation in the body must always proceed at the maximum intensity.

Changes in temperature have the same general effect on the formation of creatinine in autolyzing muscle tissue that changes in body temperature (fever) exert upon the elimination of creatinine. Creatine added to autolyzing muscle experiences the same fate as the creatine originally present, while added creatinine inhibits the reaction, or if added in sufficient quantity causes it to proceed in the opposite direction.

The muscle tissue employed in most of the autolysis experiments reported was that of the rabbit, although data are also given for the cat, dog, and hen. Some experiments, not reported but showing the usual increase in creatinine on autolysis, have also been carried out on human tissue. The results with the rabbit, cat, and hen are directly comparable. There was the same conversion of creatine to creatinine and no apparent destruction of either of these substances. In our dog autolyses, it should be noted, however, that the creatinine did not increase after a certain period and there was an appreciable drop in the total creatinine.

Experiments carried out with autolyzing human blood showed an increase in both the creatinine and the creatine, and somewhat similar observations were made in experiments with rabbit liver. It is not the object of the present paper, however, to discuss the formation of creatine or the destruction of both substances in the organism. Experiments are being conducted on nephrectomized animals which we hope will throw some light on this subject.[21] That there was no destruction of creatine or creatinine in our muscle autolyses would appear, however, to possess some significance. To what extent the changes above noted are controlled by enzymes, and in what degree they are of purely chemical nature is a matter which we feel cannot be discussed advantageously at present.

According to published data the metabolism of creatine and creatinine in the fowl differs considerably from that in mam-

[21] Myers and Fine: *Proc. Soc. Exper. Biol. and Med.*, xii, p. 41, 1914.

mals. Creatine, instead of creatinine, is excreted in the urine, and a very low concentration of creatinine in the blood has been reported by Folin and Denis.[22] From a comparative standpoint the metabolism of these substances in the fowl is of considerable interest. Our experiments here reported on autolyzing hen muscle disclose no differences from similar experiments on the rabbit and cat, nor do our observations confirm the findings of Folin and Denis of the absence of creatinine from the blood of birds. The close similarity in the autolytic relationship of creatine and creatinine in birds and mammals, and the difference in the metabolism of these two substances in the two groups of animals do, it must be admitted, appear to set definite limits to the applicability of studies *in vitro* to processes taking place in the living organism. It is possible that studies upon the hen similar to those carried out upon rabbits would disclose valuable information upon the creatine-creatinine problem in general. Until such work is done, the point above raised cannot be advantageously discussed.

Our various observations bearing upon the relation between creatine and creatinine, and the metabolic significance which they appear to us to indicate, may be summarized as follows:

1. *Muscle creatinine.* Creatinine is present in muscle tissue in higher concentrations than in other body tissues.[23] This observation strongly suggests that muscle tissue is the site of creatinine formation. It is of further interest in this connection that most of the creatine of the body is present in the muscle tissue.

2. *Muscle creatine.* Since the creatine of the muscle tissue has a relatively definite concentration for a given species of animals,[24] it is logical to assume that it is very constant for an individual animal. In the present autolysis experiments with muscle tissue it has been shown that creatine is converted to creatinine at a very constant rate. This same reaction in the body would account for the well known constancy in the excretion of creatinine.

3. *Muscle creatine and urinary creatinine.* Since the muscle creatine and urinary creatinine are constant, there must be a definite ratio between the two. This would be the equivalent

[22] Folin and Denis: *loc. cit.*

[23] Myers and Fine: this *Journal*, xxi, p. 387, 1915.

[24] Myers and Fine: *ibid.*, xiv, p. 9, 1913; xxi, p. 390, 1915.

of a daily conversion of creatine to creatinine of about 2 per cent. When creatine is administered to man or animals, there is a slight conversion to creatinine which corresponds very well with this figure;[25] and in autolyzing muscle tissue there is practically the same rate of transformation. In fevers there is a definite relationship between the rise in body temperature and the creatinine excretion;[26] and a rise in temperature has the same influence upon the formation of creatinine in autolysis.

If the experimental data here presented cannot be regarded as directly bearing upon the relationship existing *in vivo* between creatine and creatinine, they nevertheless constitute a strong set of arguments supporting our working hypothesis.

[25] Myers and Fine: *ibid.*, xvi, p. 169, 1913; xxi, p. 377, 1915.
[26] Myers and Volovic: *loc. cit.*

CONCERNING THE DISTRIBUTION OF CYANOGEN IN GRASSES, ESPECIALLY IN THE GENERA PANICULARIA OR GLYCERIA AND TRIDENS OR SIEGLINGIA.

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The investigation, here presented, is the beginning of a survey for hydrocyanic acid in the grasses of the United States.

It is stated by Greshof[1] that cyanogen, or hydrocyanic acid, is widely distributed among the *Gramineae*, or grasses. Various species of the genera *Stipa, Panicularia, Briza, Catabrosa, Elymus, Festuca, Gynerium,*[2] *Melica,*[2] *Holcus, Lamarckia, Poa, Panicum, Sorghum, Andropogon, Zea,* and *Bambusa* are now recognized as cyanogenetic. Appreciable amounts of hydrocyanic acid in the form of glucosides have been detected in various species of *Bambusa* in the young parts of the plants.[3] Couperot[4] reports analyses for hydrocyanic acid in a number of European species of *Festuca*. In only one case of these, *Festuca Poa* Kunth, has cyanogen been reported. A number of species of *Andropogon* have also been analyzed. That many of them are cyanogenetic is well known. The occurrence of cyanogen in a European species of *Panicularia, P. grandis,* has been reported by Joris-

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[1] M. Greshof: Phytochemical Investigations at Kew. *Bulletin of Miscellaneous Information*, Royal Botanic Gardens, Kew, No. 10, p. 417, 1909.

[2] A. Jorissen: L'acide cyanhydrique chez les végétaux, *Bull. de l'Acad. roy. de Belg.*, p. 1202, 1913.

[3] O. Walter: T. Krassnosselska, N. Maxinow, and W. Maltschewski: Über den Gehalt und die Verteilung von Blausäure im Bambusrohr, *Bull. Acad. St. Pétersbourg*, 1911, pp. 397–426, through *Chem. Zentralbl.*, lxxxii, p. 1864, 1911.

[4] E. Couperot: Sur quelques végétaux à acide cyanhydrique, *Jour. de Pharm. et Chim.*, series 6, xxviii, p. 542, 1908.

sen.[5] Hébert[6] found cyanogen in certain Argentine species of the genus *Stipa*, reported to be poisonous. Apparently, *Stipa Vaseyi* Scribn., the sleepy grass of the Southwest, which is reported to be poisonous to stock, has not hitherto been tested for cyanogen. No records seem to have been made of analyses of *Tridens* for cyanogen. In spite of the reports concerning so many species of grasses there has been little systematic search for this substance. There is no record of a chemical examination of any species of the majority of the genera considered in this paper. Only *Agropyrum repens*,[7] *Festuca*,[7] *Panicum* (*Digitaria*) *sanguinale*,[8] *Uniola latifolia*,[8] and species of *Bromus*[9] have been examined by the usual methods for feedingstuffs.

In Europe veterinarians have repeatedly recorded instances of poisoning of stock by *Panicularia fluitans*[10] and by *P. aquatica*, which latter was formerly known as *Glyceria spectabilis* or *Poa aquatica*.[11] The presence of cyanogen in *P. aquatica*, though recorded by Jorissen,[12] was not known to these veterinarians. The only other examination of this plant, made by Arendt and Knop,[13] does not mention cyanogen. Since the veterinarians, above cited, had observed that hay from these grasses was fed without toxic results, and since the green grass found to be poisonous was infested with the smut, *Ustilago longissima*, the toxic action was attributed by them to the fungus and not to the grass. Similarly, an unpublished case of poisoning, alleged to be due to *Epichloea typhina*, parasitic upon *Panicularia nervata*, has been reported to this Laboratory.

A list of the grasses examined by us is given in the table. The material of *Panicularia nervata* (Willd.) Ktze. (=*Glyceria ner-*

[5] Jorissen: *Bull. de l'Acad. roy. d. sc. et d. Beaux-Arts de Belg.*, series 3, viii, p. 258, 1884.

[6] A. Hébert: Recherches sur la présence de l'acide cyanhydrique chez diverses plantes (2e mémoire), *Bull. Soc. chim.*, series 3, xxxv, p. 919, 1906.

[7] C. Wehmer: *Die Pflanzenstoffe*, Jena, 1911, p. 60.

[8] P. Collier: Report of the Botanist and Chemist on Grasses and Forage Plants, *Annual Report of the Commissioner of Agriculture for the year 1878*, Washington, 1879, p. 160.

[9] E. Wolff: *Aschenanalysen von landwirthschaftlichen Produkten, Fabrik-Abfallen und wildwachsenden Pflanzen*, Berlin, 1871, pt. i, p. 42.

[10] C. Dammann: *Die Gesundheitspflege der landwirtschaftlichen Haustiere*, Hannover, 1886, p. 706. Kopke: *Vergiftungen durch Schilfgras welches Brandpilze enthielt. Mittheilungen aus der thierärztlichen Praxis im preussischen Staate*, i, p. 112, 1876. Kopke: *Vergiftungen durch Schilfgras*, iii, p. 137, 1878. J. Eriksson: Giftiges Süssgras, *Glyceria spectabilis* von *Ustilago longissima* befallen, *Ztschr. f. Pflanzenkr.*, x, p. 15, 1900.

[11] Dammann: *loc. cit.*, p. 707.

[12] Jorissen: *loc. cit.*

[13] R. Arendt and W. Knop: *Grasuntersuchungen, Die landwirthschaftlichen Versuchs-Stationen*, ii, p. 40, 1860.

vata Trin.) was collected in the vicinity of Washington, D. C., except one sample, which was collected by Mr. W. N. Suksdorf, of Bingen, State of Washington. The material of *P. canadensis* (Michx.) Ktze. (=*G. canadensis* Trin.) and *P. grandis* Nash. (=*grandis* Wats.) was collected by Dr. A. J. Eames of Cambridge, Mass. *P. septentrionalis* Bickn. (=*G. septentrionalis* Hitchc.) was collected in Hartford City, Indiana, by Mr. Charles C. Deam. *P. fluitans* Ktze., (=*G. fluitans*) and *P. pauciflora* Ktze. (= *G. pauciflora*) were collected by Mr. W. N. Suksdorf in Klickitat County, Washington. Both freshly gathered and dry material of *P. nervata* were examined. *P. canadensis*, *P. grandis*, *P. fluitans*, *P. pauciflora*, and *P. septentrionalis* were sent to the laboratory in a small amount of alcohol. Hence, the findings on this material may not be sufficiently conclusive. All samples were examined as soon as they reached the laboratory. This, of course, means that samples gathered in the immediate vicinity were examined the same day. Those sent from other places in the East were examined within two days of the time they were gathered, while the samples from the State of Washington had been gathered about a week before they were examined. The material for the examination of *Tridens* was gathered in the vicinity of Washington, D. C. The sample of *Stipa Vaseyi* was collected in Southern California while the plants were in seed, and dried before sending, the dry material having been kept about six months before examination. From the seeds young plants were grown in the greenhouse of the Department of Agriculture. These plants were examined, after freshly gathering, when they were from six to sixteen inches tall. The samples of *Festuca nutans* Willd. (=*F. obtusa* Spreng.), *Zizania aquatica* L. (=*Z. Palustris* L.), *Capriola Dactylon* (L.) Ktze. (=*Cynodon Dactylon* Pers.),. *Agropyrum repens* (L.) Beauv., *Digitaria sanguinalis* (L.) Scop. (=*Panicum Sanguinale* L.), *Homalocenchrus virginicus* (Willd.) (=*Leersia virginica* Willd.), *Homalocenchrus oryzoides* (L.) Poll. (=*Leersia oryzoides* Swartz), *Echinochloa Crus-Galli* (L.) Beauv., *Hystrix patula* Moench., and *Uniola latifolia* Michx. were collected in the neighborhood of the District of Columbia and were examined in the green state the day they were gathered. The remaining plants, given in the table, consisted of dried material, one to three years old,

from the botanical collections of the Office. *Bromus polyanthus* Scribn. was infested with the smut, *Ustilago bromivora.*

To detect cyanogen, definite weights of the plants were covered in a liter distilling flask with 300 to 400 cc. of 5 per cent sulphuric acid and distilled through a condenser into alkali, the alkali always remaining in excess. The distillation was continued until portions of 50 cc. of distillate by the Prussian blue and sulphocyanide tests showed no hydrocyanic acid present. The quantitative determinations were made in the distillate at first by the Liebig silver nitrate method. This was abandoned because it was unsatisfactory, and the remaining determinations were made by the Prussian blue method of Berl and Delpy.[14] In Experiment 15 of the table the green plants, instead of being distilled with acid soon after gathering, were macerated with distilled water and allowed to stand twenty-four hours before adding acid and distilling.

Consideration of the table shows that only *Tridens flavus, Panicularia nervata, P. grandis,* and *P. canadensis* contain hydrocyanic acid. The absence of cyanogen in *Stipa Vaseyi,* the sleepy grass of the Southwest, is of interest, both because this grass is very generally reputed to be poisonous to stock and because certain Argentine species of this genus have been found by Hébert to contain cyanogen. The discovery of cyanogen in *Tridens flavus* is interesting since it has not previously been reported in this genus. The quantities of cyanogen found are very small and after the plant is in seed none is present. Grazing stock may be observed to avoid it, perhaps because of the tar-like matter at the nodes. For these reasons, though it is a very common grass, it is not a factor of practical importance in grazing. However, it is well to bear in mind that a plant ordinarily containing small amounts of cyanogen may at times develop larger quantities, and, furthermore, that some animals may show idiosyncrasies of taste. The American species *Panicularia nervata, P. canadensis,* and *P. grandis* have not heretofore been reported to contain cyanogen. No cyanogen was found in *P. septentrionalis,* in *P. fluitans,* or in *P. pauciflora.* The examination of the

[14] E. Berl and M. Delpy: Über die quantitative colorimetrische Bestimmung kleiner Blausäure-Mengen, *Ber. d. deutsch. chem. Gesellsch.,* xliii, p. 1430, 1910.

three last named species is not, however, absolutely conclusive; since the material tested was either dry or preserved in a small amount of alcohol. The absence of cyanogen in these species is interesting; since they belong to a group of the genus rather distinct from that to which the cyanogenetic species belong. However, the accumulation of detectable quantities of cyanogen in a single species or group of species of a large genus is not unusual.

The presence of cyanogen in *Glyceria aquatica* [15] is probably the cause of toxicity in this grass. It was, probably, a mistake to attribute the toxicity to the fungus, *Ustilago longissima*,[16] cyanogen being more likely the toxic factor, since no one has yet been able to demonstrate that any species of *Ustilago* is poisonous.

The fact that the grass when infested with the fungus was sometimes observed to be non-toxic, is not conclusive evidence that the fungus was the toxic element, as shown by the studies on *P. nervata*, herein recorded, since the cyanogen content of plants varies with the different stages of growth. Moreover, it is well known that the cyanogen content varies with the conditions of growth. Plants containing cyanogen may lose much of their toxicity in drying, especially when the drying process is a slow one, as would be the case in hay-making. However, as may be seen by reference to the table, *P. nervata*, dried on standing over night, spread on the laboratory table, still retained most of its cyanogen. Furthermore, the symptoms described by the veterinarians resembled poisoning by cyanogen-containing plants, such as immature sorghum. Thus Kopke reported that the first symptoms followed almost as soon as feeding began, before a great quantity of fodder had been consumed. The animals then fell down, the temperature became subnormal, the pulse small, and the respiration rapid. When the fodder proved fatal the animals died in one and one-half hours. Eriksson reported that one and one-half hours after the grass was fed the cattle exhibited diarrhea, a subnormal temperature of 37.4° C., coldness of the skin, a tendency to lie down and withhold milk, and difficulty in getting up. On one occasion all of the animals re-

[15] Jorissen: *loc. cit.*
[16] Eriksson: *loc. cit.*

covered in one and one-half hours; on another some were so sick they had to be slaughtered.

The conditions of poisoning with *P. nervata* seem similar to those above described. In this plant, also, as above stated, a fungus was first suspected, but it seems more probable that the toxic effect is due to the large amount of cyanogen that may be found in the plant. While no careful description of the symptoms of poisoning are available, it is stated that a very small quantity of the plant was sufficient to kill stock and the course of the poisoning was exceedingly rapid, the death of calves occurring in a few minutes in some cases, in a few hours in others. While, therefore, there is abundant evidence that *P. nervata* is dangerous to stock, it is astonishing that greater losses do not occur in the Eastern United States where this plant is widely distributed. As it does not grow in dense stands, ordinarily an animal would not consume considerable quantities in a short time; and, furthermore, the commonest station is in bogs or sloughs where cattle seldom browse. In addition, the fact that the cyanogen content of plants may vary greatly must be considered.

An effort was made to test the toxicity of *P. nervata* by feeding the freshly gathered green grass to rabbits. But, even when starved, the animals did not eat the grass. Concentrated extracts from the grass could not be so prepared as to contain hydrocyanic acid. Hence, when these were fed to rabbits they proved to be innocuous.

Efforts to isolate a cyanogenetic glucoside from *P. nervata* have not yet been very successful. The methyl alcohol extract of the fresh plants was concentrated under diminished pressure with the addition of a little calcium carbonate. The residue was extracted with water and filtered. The aqueous solution was extracted with acetic ether and the extract concentrated to a syrup under diminished pressure in the presence of a little calcium carbonate. It was then filtered and set aside. After a few weeks a few crystals were obtained. The bulk of the syrup containing most of the cyanogen failed to crystallize.

The few crystals obtained were nearly insoluble in methyl alcohol, ether, benzol, chloroform, and acetic ether in the cold, though they were slightly soluble in ethyl alcohol and very soluble in water. The yield was too small to purify. When suspended in

ethyl alcohol it melted just below the boiling point of the alcohol. On hydrolysis with mineral acid cyanogen is formed and the solution reduces Fehling's reagent. The substance is rather labile, easily losing cyanogen and forming a substance crystallizing from ether in white needles melting at 59° C. (uncorrected) and having the appearance of a fatty acid.

With the knowledge of the cyanogenetic substance thus obtained it became apparent that other methods for its isolation must be developed. Water is the only solvent found to extract it completely from the plant. The watery extract was treated with saturated barium hydrate solution as long as a precipitate formed. After filtration the excess of barium was removed with carbonic acid. The resulting clear, pale solution was concentrated by freezing out the water in an ordinary ice cream freezer and removing the ice crystals by suction. In this way a fairly concentrated solution containing cyanogen was obtained. Lead acetate did not precipitate the substance. On addition of alcohol the substance was precipitated with impurities from which it could not be separated. A number of other methods were tried without success; since the substance decomposes rapidly even in neutral solutions.

SUMMARY.

Twenty-two species of American grasses were tested for cyanogen. Of these, cyanogen was found in *Tridens flavus*, *Panicularia nervata*, *Panicularia grandis*, and *Panicularia canadensis*. Three other American species of *Panicularia* examined, *pauciflora*, *fluitans*, and *septentrionalis*, did not contain it under the conditions of the examination. *Stipa Vaseyi*, sleepy grass of the Southwest, generally regarded to be poisonous, contained no cyanogen.

Results of tests for hydrocyanic acid in various grasses.

NO.	SPECIES	MATERIAL		HYDROCYANIC ACID		LOCALITY	DATE OF COLLECTION
		Weight	Part of plant	Weight			
		gm.		*mg.*	*per cent*		
1	*Stipa Vaseyi*	100	Seeds	None		California	1910.
2	" "	100	Leaves	None		California	1910.
3	" "	30	Whole plant with roots	None		Greenhouse	21, IV, 1911.
4	" "	100	Dry plants in seed	None		California	1910.
5	*Tridens flavus*	100	Plants before flowering	36.0	0.0360	Bethesda, Md.	VII, 1912.
6	" "	100	Plants after flowering	27.0	0.0270	Arlington, Va.	VII, 1912.
7	" "	100	Green plant	7.5	0.0075	Arlington, Va.	18, VII, 1914.
8	" "	100	Whole green plant	Trace		Cleveland Park, D. C.	22, IX, 1914.
9	" "	100	" " "	None		Cleveland Park, D. C.	6, X, 1914.
10	*Panicularia nervata*	100	Hay	Trace		Mt. Paddo, Wash.	7, IX, 1912.
11	" "	100	Seed	32.50	0.0325	Chevy Chase, Md.	13, VII, 1911.
12	" "	100	Roots	32.50	0.0325	" " "	13, VII, 1911.
13	" "	100	Stems	41.60	0.0461	" " "	13, VII, 1911.
14	" "	100	Entire plant	55.50	0.0555	" " "	13, VII, 1911.
15	" "	100	" "	88.00	0.0880	Riverdale, Md.	4, VII, 1912.
16	" "	100	" "	94.00	0.0940	" "	4, VII, 1912.
17	" "	75	Hay	104.00	0.1040	" "	4, VII, 1912.
18	*Panicularia pauciflora*	100	"	None		Klickitat Co., Wash.	7, IX, 1912.
19	" *fluitans*	100	"	None		" " "	7, IX, 1912.
20	" *septentrionalis*	100	Plants in seed	None		Hartford City, Indiana	15, VII, 1911.
21	" *grandis*	100	" "	22.8	0.0228	Glacialis Pond, Cambridge, Mass.	13, VII, 1911.

22	*Panicularia canadensis*	100	Plants in seed	49.0	0.0490	Glacialis Pond, Cambridge Mass.	18, VII, 1911.
23	*Festuca nutans*	100	Plants in seed	None		Chevy Chase, Md.	VII, 1911.
24	*Zizania aquatica*	100	Plants in flower	None		Anacostia River, D. C.	VII, 1912.
25	*Capriola Dactylon*	100	Plants in seed	None		Tennallytown, D. C.	VII, 1912.
26	*Agropyron repens*	100	" "	None		" "	VII, 1912.
27	*Digitaria sanguinalis*	100	" "	None		" "	VII, 1912.
28	*Homalocenchrus virginicus*	100	Entire plant	None		District of Columbia	VIII, 1912.
29	*Homalocenchrus oryzoides* (L)	100	" "	None		" "	VIII, 1912.
30	*Echinochloa Crus-Galli*	100	" "	None		" "	VIII, 1912.
31	*Hystrix patula*	100	" "	None		" "	VIII, 1912.
32	*Uniola latifolia*	100	" "	None		" "	VIII, 1912.
33	*Calamovilfa longifolia* (Hook) Hack.	100	Dry plants	None		Hugo, Colorado	19, VII, 1909.
34	*Bulbilis dactyloides* (Nutt.) Raf.	100	" "	None		Hugo, Colorado	19, VII, 1909.
35	*Bromus polyanthus*	100	" "	None		Mt. Carbon, Col.	20, VII, 1909.
36	*Andropogon saccharoides* Swartz	100	Entire plant	None		Kansas	VIII, 1912.

THE AMINO-ACID CONTENT OF CERTAIN COMMERCIAL FEEDINGSTUFFS AND OTHER SOURCES OF PROTEIN.

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In the spring of last year it was suggested to me by Dr. Kastle that it might be of interest to study the distribution of the nitrogen of the amino-acids in the mixed proteins of commercial feedingstuffs and other sources of protein. Such information might prove of interest as throwing light on the constitution of mixed proteins and might also prove of great importance and value in the practical problems of animal nutrition.

The recent work of Mendel and Osborne and also that of McCollum, in the feeding of isolated proteins, has shown that certain amino-acids are essential for growth and maintenance. Their work has shown that the presence of the amino-acid, lysine, is necessary for normal growth. Likewise they have shown that tryptophane is necessary in the amino-acid make-up for maintenance, and more recently cystine has been found to play an important part in normal nutrition. A new light has been thrown on the subject of nutrition by these masterly investigations, and there is no doubt that many of the older ideas concerning animal feeding will have to be abandoned. With such information at hand nutrition experiments will no doubt be placed on a more rational basis than heretofore. In fact, it is probably not too Utopian to expect that protein feeding in the future will be based rather on the amino-acid make-up than on the results of past feeding experiments.

With such ideas as these in mind a systematic study of the nitrogen distribution in commercial feedingstuffs and a few other sources of protein has been undertaken.

The methods for isolating the amino-acids yielded by hydrolyzed proteins, as originally proposed by Kossel and Fischer, have given us most of our present knowledge of the composition and chemical nature of the protein molecule. The application of these methods, however, is somewhat limited on account of the large amount of material necessary, and chiefly for the reason that the methods for determining most of the amino-acids are not quantitative. The most careful work accounts for only about one-half to two-thirds of the protein molecule. This is, however, little more than we would expect when we consider the many possible sources of error due to the manipulation necessary in the procedure.

The method used in determining the nitrogen distribution in the results herein presented is that described by Van Slyke,[1] with a few minor changes which will be noted later. This method is based, not on the isolation of the amino-acids, but on the determination of their characteristic chemical groups.

The samples of commercial feedingstuffs and other protein substances were ground so as to pass through a 40 mesh sieve. The fat was removed by extracting the finely ground sample with ether. This removal of fat was found advantageous in that it obviated foaming during the hydrolysis and facilitated the measuring of an aliquot portion later in the analysis, by giving a clear liquid.

The hydrolysis was carried out with 20 per cent hydrochloric acid in a tared flask under a Hopkins reflux condenser. At intervals of six or eight hours the hydrolysis was stopped, the flask and contents were cooled, and portions of 1 cc. withdrawn by a pipette. These portions were diluted to 10 cc., and the amino-nitrogen was determined in the Van Slyke apparatus.[2] The hydrolysis was continued until the amount of amino-nitrogen was constant. The flask and contents were weighed after withdrawing the sample, these weights serving to detect concentration in the solution by loss of vapor. A considerable amount of fiber was left in some of the samples after the hydrolysis was completed. This was carefully filtered off and the resulting clear solution of amino-acids used for the analysis.

[1] D. D. Van Slyke: this *Journal*, x, pp. 15–55, 1911–12.

[2] Van Slyke: *ibid.*, xii, pp. 275–284, 1912.

The solution of hydrolyzed protein was concentrated under diminished pressure in order to remove as much of the hydrochloric acid as possible. The syrupy residue was taken up with warm water and transferred to a measuring flask of 250 cc. capacity. Aliquot portions were then taken for the determination of total nitrogen by the Kjeldahl method, on the basis of which the final results were calculated.

The few minor changes in the original method were notable as regards certain dilutions which facilitated the handling of the several fractions. All determinations were made in duplicate. The results of these determinations are given in Table I.

As far as could be ascertained the presence of sugars resulting from the hydrolysis of starch and other carbohydrates in some of the original samples introduced no complications and interfered in no way with the analysis; neither did the gums and resins contained in the commercial feedingstuffs of plant origin. All results are corrected for the solubility of the bases.

An examination of the results given in the table shows many marked individual peculiarities of the different mixed proteins. Among such peculiarities may be mentioned the absence of histidine in distillers' dried grains and in the cow-pea; the absence of non-amino-nitrogen, representing proline and oxyproline, in wheat bran and the maize kernel. The relatively large amount of lysine present in the soy bean, distillers' dried grains, wheat bran, dried blood, maize kernel, hemp-seed, and sunflower seed is especially noteworthy. In contrast to this we have an absence of lysine in rice,[3] oat grain, rolled oats, and barley grain. The mono-amino-acids constitute, in most cases, about one-half of the amino-acids present. The high ammonia content and the low lysine content of gluten (wheat) and gluten flour is marked. Similar results have been obtained in the analysis of gliadin by Van Slyke.[4]

The high arginine content of the peanut, black walnut, and hickory nut as compared with the relatively small amount of arginine in the pecan is noteworthy. The reverse of this is found in the histidine content of these nuts. Many other peculiarities and interesting facts presented by the results could be pointed

[3] See footnote under Table I.

[4] See note 1.

TABLE I.

Distribution of nitrogen in various protein substances.

	AMMONIA N	MELANIN N	CYSTINE N	ARGININE N	HISTIDINE N	LYSINE N	AMINO N OF FILTRATE: Mono-amino-acid N	NON-AMINO N OF FILTRATE: Proline, oxy-proline, tryptophane, etc.	TOTAL
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Soy bean	12.97	3.69	1.52	15.52	2.60	7.02	48.76	7.12	99.20
Distillers' dried grains	13.06	8.21	3.02	11.27	0.00	4.79	50.68	10.66	101.70
Cottonseed meal	14.06	6.27	2.74	12.77	7.57	1.94	45.02	7.49	97.86
Cow-pea	11.83	9.57	6.74	15.98	0.00	3.56	46.70	0.58	94.96
Wheat bran	9.67	13.75	5.96	12.53	3.84	4.04	49.95	0.00	99.74
Maize kernel	4.63	7.00	4.06	16.19	4.45	8.53	49.69	0.00	94.55
Hemp-seed	9.93	4.15	2.05	21.38	3.01	6.71	44.20	5.28	96.73
Rice*	10.23	9.98	6.97	11.94	3.18	0.00	38.83	15.90	97.03
Sunflower seed	15.42	5.73	2.98	16.80	4.56	4.86	45.32	5.27	100.92
Rolled oats	13.12	2.60	5.22	12.12	10.54	0.00	46.99	12.68	103.27
Oat grain	13.31	2.97	4.48	11.42	9.58	0.00	43.49	11.29	96.54
Sprouted oats	13.18	2.40	5.32	11.26	9.61	0.70	41.61	12.48	96.56
Barley grain	16.19	2.87	4.38	8.65	6.70	0.00	44.16	18.37	101.32
Rye grain	15.00	1.54	2.20	10.49	10.48	1.24	37.96	21.63	100.52
Swift's digester tankage	10.03	6.88	2.46	12.34	2.18	2.50	54.73	9.01	100.13
Armour's dried blood	6.19	5.69	2.02	7.72	8.37	9.97	51.53	3.94	95.44
Unroasted peanut	10.93	4.36	0.81	20.82	6.13	5.31	52.36	1.40	99.12
Black walnut	10.71	4.53	1.27	23.77	5.98	3.49	45.01	3.12	97.90
Shellbark hickory nut	9.47	6.59	1.58	24.24	6.66	3.37	43.25	7.48	103.61
Pecan	9.43	6.21	2.87	6.91	21.91	3.25	42.28	7.89	100.75
Gluten flour	22.99	1.31	2.12	8.86	5.18	0.40	49.19	7.67	97.72
Gluten (wheat)	22.53	1.01	1.91	7.61	5.57	0.51	49.05	9.76	97.95

* Dr. Van Slyke has been kind enough to call my attention to the fact that possibly there is an error in the cystine determinations in the rice, barley, and oats of the above series, as the result of which the cystine in these grains has been found to be too high and the lysine correspondingly low. An effort is being made to settle this point at the present time.

out, but the above will suffice to show the marked differences in amino-acid content shown by these commercial feedingstuffs and various other sources of proteins.

In conclusion, I desire to acknowledge my indebtedness to Dr. Joseph H. Kastle for valuable suggestions during the progress of this investigation.

THE INFLUENCE OF THE COMPOSITION AND AMOUNT OF THE MINERAL CONTENT OF THE RATION ON GROWTH AND REPRODUCTION.[1]

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That the sodium, potassium, calcium, magnesium, phosphate, chlorine, and sulphate ions play an essential rôle in living protoplasm is a fact generally accepted. That the ion-protein compounds determine the peculiar properties of the membranes of living tissues is highly probable,[2] and the constancy of composition of these combinations of ions with proteins in most of the body tissues is maintained only when the concentrations of the several ions in the liquid medium, the blood and lymph, remain constant within certain limits. The sensitiveness of the heart muscle to "unbalanced" salt solutions,[3] and of eggs developing in sea water to which a single basic ion as magnesium or potassium has been added in excess[4] suggests that even slightly abnormal relationship between certain ions in the blood, if maintained for a prolonged period, may prove detrimental to the higher organisms.

Students of nutrition have until recently laid emphasis only upon the need of an adequate supply of each of the essential inorganic constituents of the diet, the assumption being made

[1] Published with the permission of the Director of the Wisconsin Experiment Station.

[2] J. Loeb: *The Dynamics of Living Matter*, New York, 1906. For an extensive review of the literature relating to the rôle of inorganic ions in physiological processes, see T. B. Robertson: *Ergebn. d. Physiol.*, x, p. 216, 1910.

[3] W. H. Howell: *Am. Jour. Physiol.*, ii, p. 47, 1899; vi, p. 181, 1902, gives the literature and a discussion of the part played by the several ions in their action on the heart.

[4] C. R. Stockard: *Arch. f. Entwcklngsmechn. d. Organ.*, xxiii, p. 249, 1907.

that an excess of any element over what is needed can be easily eliminated through excretory channels. The question as to the possible injury which might result from taking for long periods a mineral mixture of unsatisfactory composition did not arise as a serious one until an attempt to nourish calves to maturity on rations derived from a single plant source was made at the Wisconsin Experiment Station.[5] Efforts were made at that time to modify the mineral content of a ration derived from the wheat plant, which was an inadequate source of nutriment, by salt additions in order to imitate the base content carried by the highly successful ration derived solely from the corn plant.

This problem is an important one in experimental work involving nutrition during a long period on any monotonous diet. We have reported our experience[6] with rats which we caused to derive the organic portion of the ration entirely from the wheat kernel, which indicated that making salt additions to wheat so that the total mineral content of the ration was similar to milk, benefited the animals in a marked degree. With rations made up of casein, carbohydrates, fats, and a salt mixture, we had much better success in inducing growth in young rats with salt mixtures made in imitation of the inorganic content of milk or of egg, than with a mixture made in imitation of the inorganic content of the wheat kernel.

Osborne and Mendel have likewise reported their conviction that the composition of the mineral content of the diets made up of isolated food ingredients was a most important factor influencing the ability of young rats to grow.[7]

Emphasis has also been laid upon the balance between the base- and acid-forming elements as a factor of importance in nutrition,[8] but convincing experimental evidence is wholly

[5] E. B. Hart, E. V. McCollum, H. Steenbock, and G. C. Humphrey: *Wisconsin Agricultural Experiment Station, Research Bull.*, No. 17, p. 178, 1911.

[6] E. V. McCollum and M. Davis: Proc. Soc. Biol. Chemists, this *Journal*, xiv, p. xl, 1913.

[7] T. B. Osborne and L. B. Mendel: *Carnegie Institution of Washington, Publication No. 156*, pt. ii, 1911.

[8] J. H. Kastle: *Am. Jour. Physiol.*, xxii, p. 284, 1908. H. C. Sherman and A. O. Gettler: this *Journal*, xi, p. 323, 1912. E. B. Forbes: *Ohio Agricultural Experiment Station, Bull.*, No. 207, 1909.

lacking to demonstrate that diets acid within moderate limits produce physiological disturbances in normal individuals. McCollum reported satisfactory growth from an early age, and normal reproduction in rats fed exclusively on boiled egg yolk,[9] which is probably the most acid of our naturally occurring foodstuffs. Steenbock, Nelson, and Hart,[10] studied the effect of an acid diet on the retention of nitrogen in calves, and established the fact that even a high acidity does not interfere with growth on a milk diet plus mineral acids.

We have attempted to throw light on the numerous questions relating to the effects of the composition and quantity of the mineral content of the ration by an elaborate study which is still in progress. The data reported in this paper, while they do not answer with finality the main questions which the experiments were designed to answer, have brought to light several new and interesting facts.

PLAN OF THE EXPERIMENTS.

We sought a ration the organic constituents of which we believed perfectly satisfactory for complete nutrition, but which was so low in mineral content that it would not suffice for growth at the normal rate. By the systematic addition of salt mixtures of suitable composition to this ration we hoped to fix the following points:

1. With the mineral content of constant composition, what is the lowest level of intake which will serve to maintain growth at the normal rate, to maturity?
2. Will the lowest plane of intake of this particular inorganic mixture which will just suffice for growth at the normal rate also induce normal reproduction?
3. The effect of various planes of intake of the salt mixture carried by our ration on reproduction.
4. At what plane of intake is growth or reproduction depressed?
5. The effect of acidity and of alkalinity of the diet on growth and reproduction.

[9] E. V. McCollum: *Am. Jour. Physiol.*, xxv, p. 127, 1909–10.

[10] H. Steenbock, V. E. Nelson, and E. B. Hart: this *Journal*, xix, p. 399, 1914.

General animal husbandry experience with which the results of Osborne and Mendel were in accord led us to believe at the time these experiments were instituted that 18 per cent of protein was near the optimum for growth. It is not possible to fulfill all the requirements we have enumerated in a single ration carrying 18 per cent of protein, since every protein contains sulphur, which is a source of acid, and when even small amounts of phosphate and chlorine ions are introduced, the bases necessary to make the ration a neutral one always produce fairly high total base content—in fact too high for us to discover the minimum requirements of rats for the inorganic elements with a neutral diet. We have since discovered that with proteins from certain sources the protein content of the ration may be reduced far below this level, and normal growth and reproduction still be attained.[11] These observations make possible the employment of rations with lower potential acidity with hope of successful nutrition.

The ration must consist of materials which can be had in large quantities at reasonable cost, and above all their composition must be so uniform that we can depend upon the close duplication of our rations as their preparation is repeated. Such a ration we hoped to find in a mixture of a small amount of milk powder, with purified sucrose, casein, butter fat, agar-agar, and dextrin. By lowering the content of milk powder, the only ingredient which carries appreciable amounts of bases, we could regulate the base content of the diet at will. Casein, however, contains 0.8 per cent of sulphur and 0.85 per cent of phosphorus, which makes one gram a potential source of about 1.03 cc. $\frac{N}{1}$ solution of acid. We began the present series of feeding experiments with rats, employing as a basal ration a mixture of foodstuffs which was decidedly acid. It consisted of:

	gm.
Milk powder (Merrill-Soule)	10
Casein	15
Butter fat	5
Agar-agar	2
Dextrin	53
Sucrose	15

The base content of the ration was derived entirely from 10 grams of milk powder. For purposes of calculation we have

[11] McCollum and Davis: *ibid.*, xx, p. 415, 1915.

accepted the data of Forbes, Beegle, and Mensching[12] for dry skimmed milk, which assign to it the following content of inorganic elements.

	K	Na	Ca	Mg	S	Cl	P	Alkalinity $\frac{N}{1}$ solution cc.
100 gm. dry skimmed milk	1.272	0.488	1.336	0.146	0.357	0.953	0.979	19.91

Except where salt additions were made the acidity or alkalinity of the rations are expressed by the difference between the acidity of the 15 grams of casein (15.5 cc. $\frac{N}{1}$ solution) and the alkalinity of the milk powder employed. The dextrin employed contained 0.08 per cent of ash exclusive of SiO_2.

The character of the rations employed in this series of experiments is shown in Table I, together with the degree of acidity or alkalinity of each. The rations range from an acidity of 14.31 cc. to an alkalinity of 102.09 cc. $\frac{N}{1}$ solution per 100 grams. In modifying the balance between the acid and basic radicals, additions of the salt mixture described in Table III were employed, the content covering a range of 1 gram to 20 grams per 100 grams of ration. The approximate content of the elements in each of the rations is shown in Table IV.

Distilled water only was provided to the rats in these experiments and a small amount of iodine was given once each week in the form of a KI-I solution in the drinking water.

The records of the growth and reproduction of the twenty groups of rats discussed in this paper are shown in Charts I to XI, and the records of reproduction and certain details regarding the histories of the young are exhibited in tabular form in Table II.

An inspection of the charts reveals a surprising uniformity of growth in all the animals of the various groups except the two receiving the highest additions of salts (Chart VI), which were stunted by the high salt content. The records establish, first of all, that *provided the other factors in the ration are adequate, young rats can grow normally and remain in apparent good health on rations whose base content varies widely in amount.* Ration 218–6 (Chart I) contained only about 0.2 gram of total bases per

[12] E. B. Forbes, F. M. Beegle, and J. E. Mensching: *Ohio Agricultural Experiment Station Bull.*, No. 255, 1913.

100 grams. While this group did not grow quite as rapidly as did those receiving more bases, yet there was no pronounced stunting. At the other extreme is Lot 226 (Chart VI) whose ration contained 8 per cent of the salt mixture and a base content of 2.40 grams per 100 of ration. This base content they have stood without signs of disturbance during five months.

In the second place our data confirm the conclusion reached from the observation that egg yolk as a restricted diet was not deleterious as the result of its acid character.[13] *Four of the rations employed in this work* (Charts I and II) *were highly acid yet growth and well-being were not markedly interfered with.* The data in this paper alone do not show whether it was the acidity or the low base content which was the cause of failure to reproduce, but from the fact that egg yolk alone as a food supply admits of reproduction it seems probable that the low base content is the determining factor.

It was a surprise to us that all lots failed to repeat reproduction at normal intervals. This failure is certainly the result of some factor other than the character of the mineral content and must remain unexplained for the present.

In Lot 205 (Chart II) especially, copulation was frequently observed during several months, yet not a single young one was produced by the entire group of females. We tested Lots 205, 200, 201, and 204 with normally fed males placed with the experimental females during the day for several weeks, but without succeeding in securing young from any of them. The sporadic pregnancies in Lots 218, 206, 224, and 209 indicate that the failure in reproduction did not rest with the males. Whether fertilization took place, followed by the early death of the ovum, we have not yet determined.

Another conclusion which we have previously emphasized we are forced to accept as a result of the observations recorded in this paper, *viz*: *Growth to the normal adult size at the usual rate and continued well nourished appearance is not sufficient evidence that a ration is fully adequate. Only when normal reproduction and rearing of the young is repeated at normal intervals can a ration be said to be physiologically sufficient.*

[13] McCollum: *loc. cit.*

One may logically inquire why, if these rats have shown themselves with respect to growth independent of the composition and amount of the inorganic content in so marked a degree, is such great improvement noted as the result of adding suitable salt mixtures to rations restricted to the corn or wheat kernel,[14] or to other rations made up of purified food substances? The fact cannot be disputed, and we cannot, with the data available, give more than a tentative explanation.

In a ration derived entirely from the corn kernel there are two factors which are unfavorable to growth. The protein cleavage products from corn can be retained for growth only to the extent of 23 to 24 per cent of the amount ingested, which gives them a value of about one-third that of an equivalent amount of milk proteins.[15] The mineral content of the corn kernel is unfavorable for growth. It seems not improbable that these two factors together are sufficient to prevent growth, which, however, can go on when the mineral content is corrected.

The problem of the influence of the unfavorable mineral content of wheat is complicated by the presence of two deficiencies; *viz.*, the poor character of the proteins and the deficiency of the unknown accessory carried by butter fat, egg fat, corn oil,[16] etc. This is made evident by experiments which were an outgrowth of those just reported. In Lot 211 (Chart IX) we fed a ration designed to show the effects of replacing a part of the dextrin and casein of the ration of Lot 200, by wheat. The salt addition was designed to correct the mineral content of the wheat. The fact that four litters have been nourished to vigorous independence by one female on this ration proves this to be an adequate diet.

In Lot 223 (Chart X) we replaced the milk powder of Ration 211 by casein, dextrin, and salts, in proportions to cause as little change as possible in the known constituents of the ration. The records of eight females show that this ration is as good as, if not better than 211.

The next step was to eliminate in turn each of the three additions to the wheat in the most successful ration (223B); *viz.*, butter fat, casein, and salt mixture. Without the butter fat there was

[14] E. B. Hart and E. V. McCollum: this *Journal*, xix, p. 373, 1914.
[15] McCollum: *ibid.*, xix, p. 323, 1914.
[16] McCollum and Davis: *ibid.*, xxi, p. 179, 1915.

normal growth (Lot 223, Chart XI) and in the case of one of two females a single litter of young was produced. The young lived from eleven to twenty days. After four months the animals were in a miserable condition.

When wheat and wheat gluten supplied the entire protein content of the ration (Lot 24B, Chart IX) there was a little improvement over the group just described (Lot 223, Chart XI), but nutrition was far from normal.

Our results with wheat, casein, and butter fat, without salt additions have been so unsatisfactory that we withhold judgment on them until a sufficient number of individuals pronounce them negative. We can only say that in our rather extensive feeding of wheat-containing rations we have not had success without a correction of the mineral content of the ration. On the other hand, the addition of salts alone to a ration derived entirely from wheat or wheat and wheat gluten gives a diet which is a wonderful improvement over the grain alone, yet such rations give less than half normal growth, and do not suffice for prolonged maintenance. Such results present an interesting problem: Is there an interdependence between an unfavorable mineral content and other factors in the diet which causes the inorganic deficiencies to be more pronounced in some cases than in others? The answer to this question will come from the application of definite knowledge of all the essentials of a successful diet to work with rations composed of purified foodstuffs.

In Table I is given the composition of the rations employed, together with the degree of acidity or alkalinity of each, and the character of the salt additions when such were made.

The content of mineral elements carried by the rations discussed in this paper is shown in Table IV. The figures express grams of elements per 100 of ration.

TABLE I.

NO. OF RATION	COMPOSITION OF RATION	REACTION OF RATION $\frac{N}{1}$ SOLUTION PER 100 GM. OF RATION		SALT ADDITIONS (SEE TABLE III)
		Acidity	Alkalinity	
	per cent	*cc.*	*cc.*	
218–6	Milk powder† 6, casein 15, butter fat 5, dextrin 57, sucrose 15, agar-agar 2	14.31		None.
218–7	Milk powder 7, casein 15, butter fat 5, dextrin 56, sucrose 15, agar-agar 2	14.11		None.
218–8	Milk powder 8, casein 15, butter fat 5, dextrin 55, sucrose 15, agar-agar 2	13.91		None.
205	Milk powder 10, casein 15, butter fat 5, dextrin 53, sucrose 15, agar-agar 2	13.51		None.
204	Like 205, but with salt addition	7.73		1 gm. mixture.
203	" " "	1.95		2 " "
202	" " "		3.85	3 " "
201	" " "		9.61	4 " "
200	" " "		15.39*	5 " "
224	" " "		21.17	6 " "
226	" " "		32.73	8 " "
230	" " "		78.97	16 " "
238	" " "		102.09	20 " "
206	" " "		7.50	2.9 " Ca lactate.
208	" " "		12.57	2.5 " Na citrate.
209	" " "		8.60	2.4 " K citrate.
210	" " "		12.81	3.1 " Mg citrate.
211	Milk powder 10, casein 10, wheat 64, salt mixture 3.6, dextrin 7.4, butter fat 5		3.23	See Chart IX.
24B	Wheat 50, wheat gluten 17, dextrin 7, butter fat 5, salt mixture 24		83.12	See Chart IX.
223B	Wheat 64, casein 13.4, dextrin 12.8, butter fat 5, salt mixture 4.8		17.90	See Chart X.
223	Same as 223B but with dextrin replacing the butter fat		17.90	See Charts X and XI.

* The alkalinity of 100 gm. of a mixture of milk powder 53 gm. and dextrin 47 gm. (which mixture contains 18 per cent of protein) is equivalent to 10.55 cc. of $\frac{N}{1}$ solution (Forbes). The degree of alkalinity of such a milk ration falls between Nos. 201 and 200.

† The milk powder contained 34 per cent of protein.

TABLE II.

No. of lot	No. of females	No. of litters of young born, and no. of young ( )	Litters dead next morning	Young alive at 10 days of age	Young alive at 21 days of age	No. of 2nd litters	No. of 3rd litters	Young able to go on mother's ration	Remarks
224	4	1 (eaten by other rats)							
200	4	3 (13 young)	0	12	12	0	0	12	Seven young were discontinued in good condition. Five are still on this ration.
200 2nd	5 gene	1 ration	1	0	0	0	0	0	
201	5	9 (29 young)	1	19	9	3	1	3	The young from this lot had repeated convulsions at 17–21 days of age.
202	7	*3 (13 young)	0	6	0	0	0	0	Young manifested same symptoms as did those in Lot 201.
203	6	3 (16 young)	0	4	4†	1	0	2	
204	9	6 (22 young)	0	21	10	2	0	7	One young in a serious nutritive condition recovered on ration 223B. Three young were discontinued in good condition.
205	10	0	0	0	0	0	0	0	
218	4	1	1	0	0	0	0	0	
206	6	1	0	1	0	0	0	0	
208	5	2 (10 young)	0	10	6‡	0	0	0	
209	5	1	1	0	0	0	0	0	
210	4	0	0	0	0	0	0	0	

* One female died at parturition.

† One young completely recovered on ration 223B.

‡ The young which survived were from a second litter born after an additional iron supply was added to the ration.

TABLE III.

Composition of the salt mixture employed in the rations.

		Na	K	Ca	Mg	Cl	P	S	Fe
	gm.								
NaCl	12.31	4.84				7.47			
K_2HPO_3	28.08		12.6				4.99		
$CaH_4(PO_4)_2.H_2O$	0.74			0.11			0.18		
$MgSO_4$ (Anhydrous)	1.56				0.31			0.42	
Mg citrate	5.75				0.58				
Na citrate (anhydrous)	3.12	0.83							
Ca lactate	46.80			6.78					
Fe'' lactate	1.64								0.319
	100.00	5.67	12.6	6.89	0.89	7.47	5.17	0.42	0.319

TABLE IV.

NO. OF RATION	K	Na	Ca	Mg	S	Cl	P	Fe
218–6	0.0763	0.0293	0.0801	0.0087	0.1414	0.0572	0.1862	0.00014
218–7	0.0890	0.0341	0.0935	0.0102	0.1450	0.0667	0.1960	0.00016
218–8	0.1017	0.0390	0.1069	0.0117	0.1485	0.0762	0.2058	0.00018
205	0.1272	0.0488	0.1336	0.0146	0.1632	0.0953	0.2179	0.00023
204	0.2532	0.1055	0.2025	0.0235	0.1674	0.1700	0.2696	0.00340
203	0.3792	0.1622	0.2714	0.0324	0.1716	0.2447	0.3213	0.00660
202	0.5052	0.2189	0.3403	0.0413	0.1758	0.3194	0.3730	0.00980
201	0.6312	0.2756	0.4092	0.0502	0.1800	0.3941	0.4247	0.01300
200	0.7572	0.3323	0.4781	0.0591	0.1842	0.4688	0.4764	0.01620
224	0.8832	0.3890	0.5470	0.0680	0.1884	0.5435	0.5281	0.01920
226	1.1352	0.5024	0.6848	0.0858	0.1968	0.6929	0.6390	0.02580
230	2.1432	0.9560	1.2360	0.1570	0.2229	1.2905	1.0526	0.05140
238	2.6472	1.1828	1.5116	0.1867	0.2397	1.5512	1.2199	0.06420
206	0.1272	0.0488	0.5541	0.0146	0.1632	0.0953	0.2179	0.00023
208	0.1272	*0.6488*	0.1336	0.0146	0.1632	0.0953	0.2179	0.00023
209	*0.9900*	0.0488	0.1336	0.0146	0.1632	0.0953	0.2179	0.00023
210	0.1272	0.0488	0.1336	0.3308	0.1632	0.0953	0.2179	0.00023
211	0.6320	0.2290	0.3750	0.0970	0.2150	0.3780	0.3960	0.03900
24B	2.5650	1.2200	2.3780	0.1090	0.3120	1.8780	1.4150	0.02100
223B	0.7830	0.2970	0.4580	0.0950	0.1340	0.4670	0.3780	0.04300
223	0.7830	0.2970	0.4580	0.0950	0.1340	0.4670	0.3780	0.04300

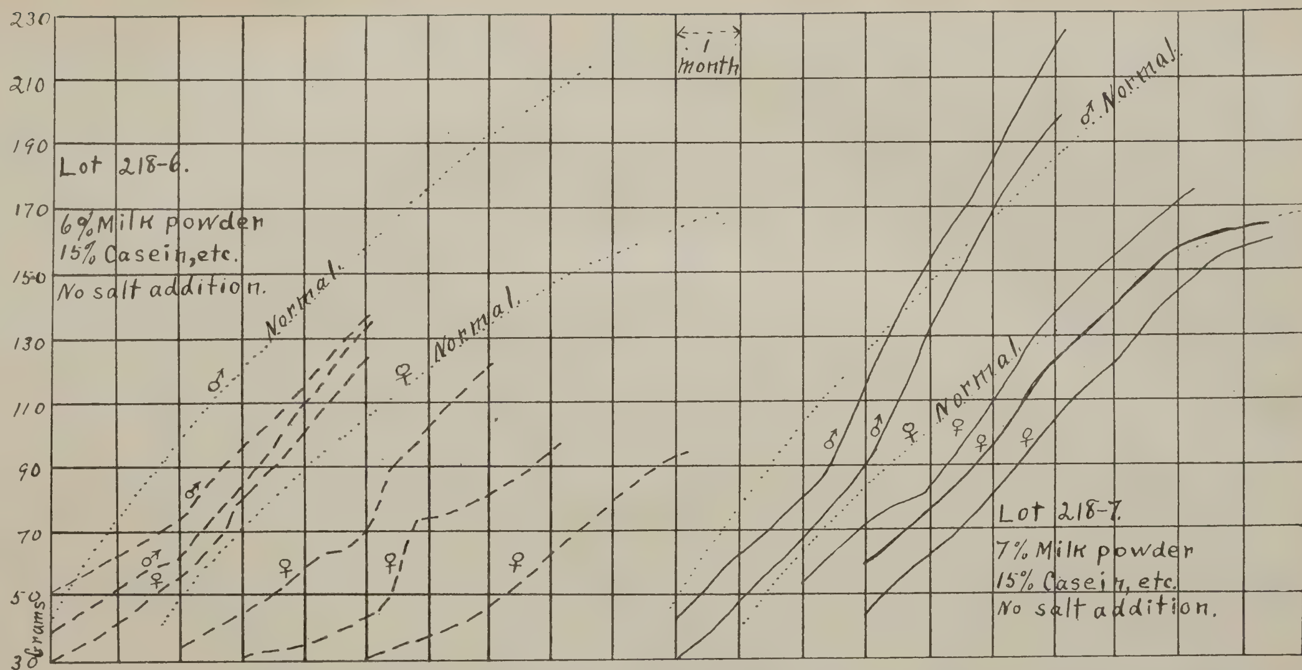


CHART I. Shows normal growth in rats which derived their entire supply of bases from 6 per cent of milk powder in their ration (Lot 218–6).

The ration was decidedly acid in character in addition to carrying a very low base content (see Tables I and IV). At the present time after five months on this diet they are in good condition.

The same thing is illustrated by the curves for Lot 218–7 (unbroken curves). When the ration is otherwise satisfactory, young rats can grow on a surprisingly low base supply. The preponderance of acid radicals in the diet does not interfere with growth.

RATION 218–6	per cent	RATION 218–6	per cent	RATION 218–7	per cent	RATION 218–7	per cent
Milk powder	6	Agar-agar	2	Milk powder	7	Agar-agar	2
Casein	15	Dextrin	57	Casein	15	Dextrin	56
Butter fat	5	Sucrose	15	Butter fat	5	Sucrose	15

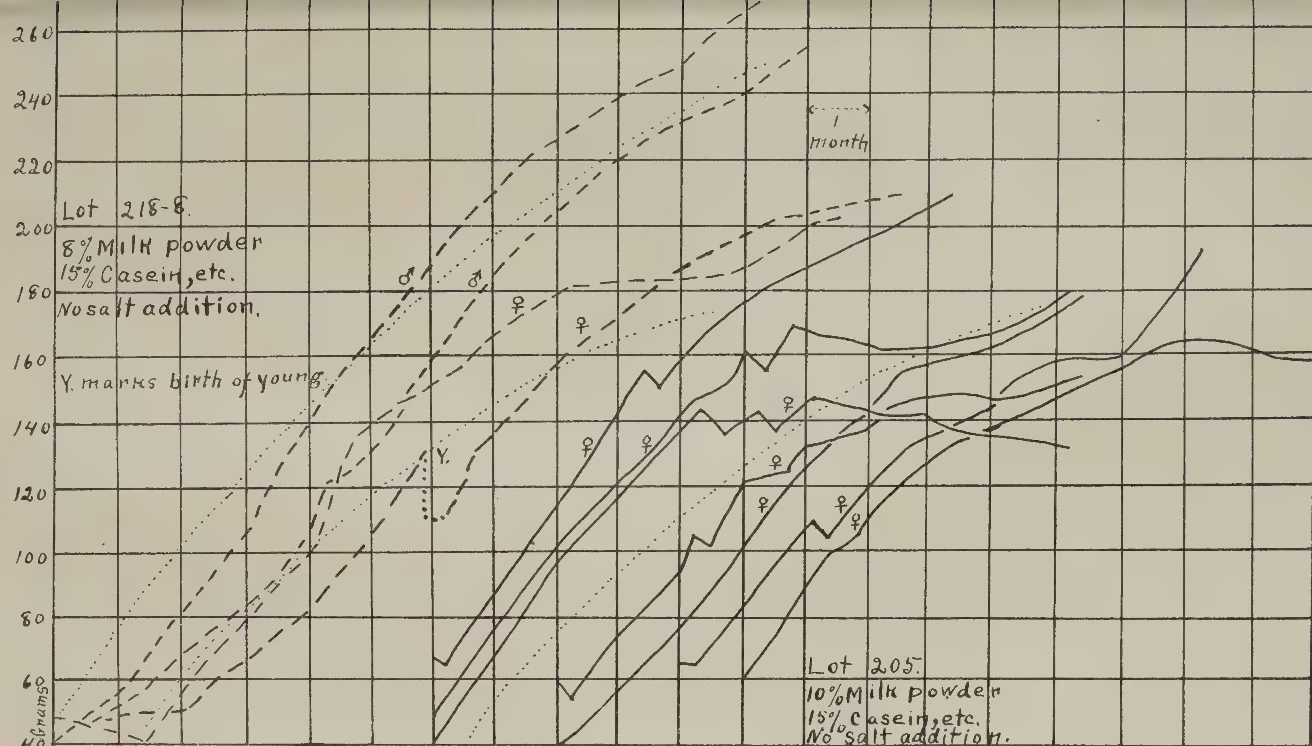


CHART II. Shows the records of animals which made normal growth on rations in which the supply of bases was furnished by 8 per cent of milk powder (Lot 218–8) and 10 per cent of milk powder (Lot 205) respectively. Ration 218–8 was acid to the extent of 13.9 cc., and 205 to the extent of 13.51 cc., of normal solution per 100 gm. From the nine females in these two groups, but a single litter of young was born, and these young were dead within a few hours. Normal growth to maturity is not a sufficient criterion of normal nutrition. Only when normal reproduction repeated at normal intervals is attained, can it be said that a ration is entirely adequate.

RATION 218–8	per cent	RATION 218–8	per cent	RATION 205	per cent	RATION 205	per cent
Milk powder	8	Agar-agar	2	Milk powder	10	Agar-agar	2
Casein	15	Dextrin	55	Casein	15	Dextrin	53
Butter fat	5	Sucrose	15	Butter fat	5	Sucrose	15

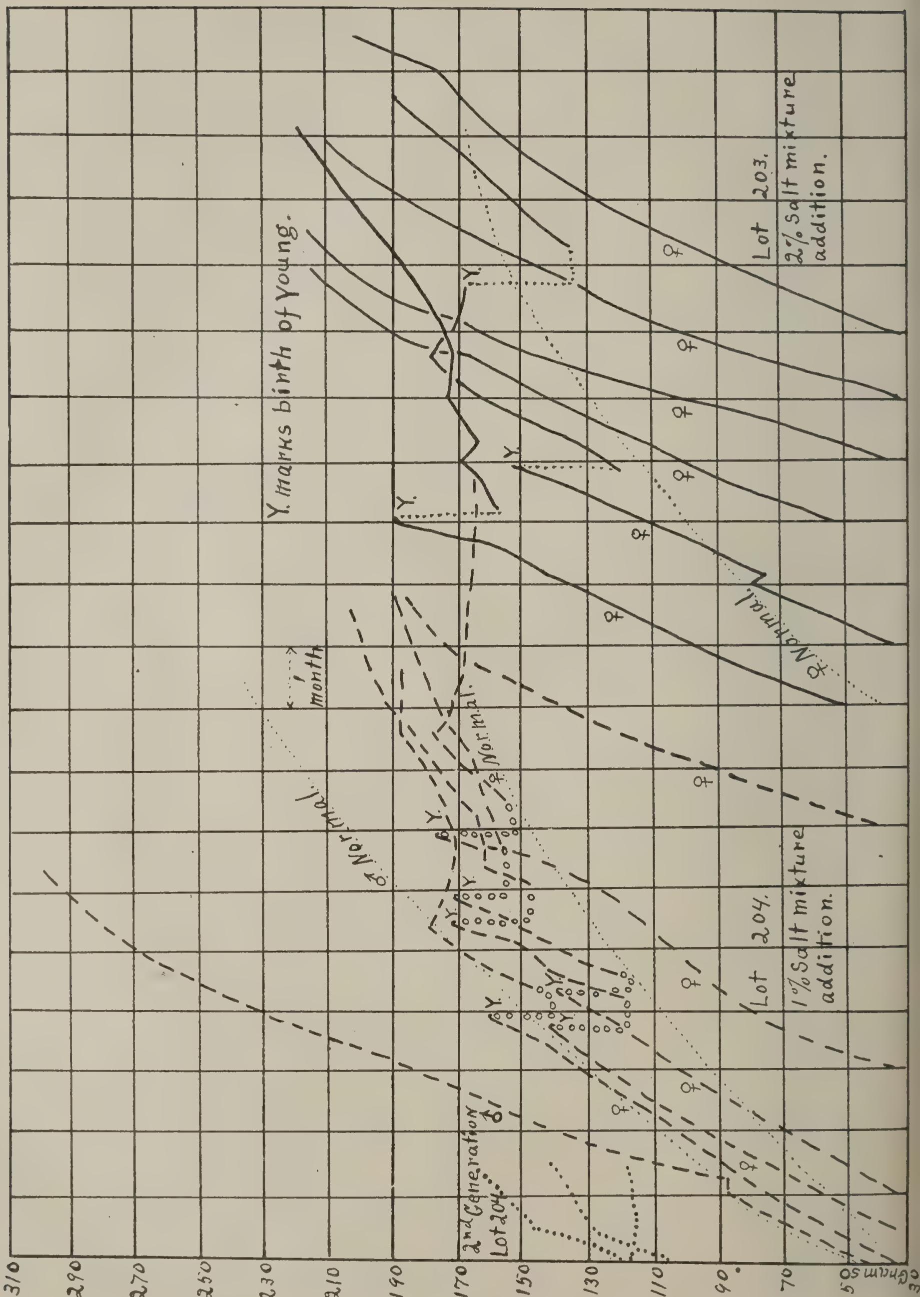

Y. marks birth of young.
Lot 203.
2% Salt mixture addition.
Lot 204.
1% Salt mixture addition.
2nd Generation Lot 204.
Normal.
month
310
290
270
250
230
210
190
170
150
130
110
90
70
50 Grams
30

Chart III. Shows the marked influence on reproduction which resulted from the addition of 1 per cent of the salt mixture described in Table III (Lot 204), and of adding 2 per cent of the same mixture (Lot 203) to the ration employed with Lot 205 (Chart II). With this ration without these salt additions no reproduction could be secured. It should be emphasized that the mortality of the young in both these lots, prior to weaning time, was high (see Table II).

These rats should each have produced about four litters of young during the period covered by their curves if they had been given a suitable diet. The failure to do this indicates what could not be seen from their growth curves or appearance, that the ration is inadequate in some respect.

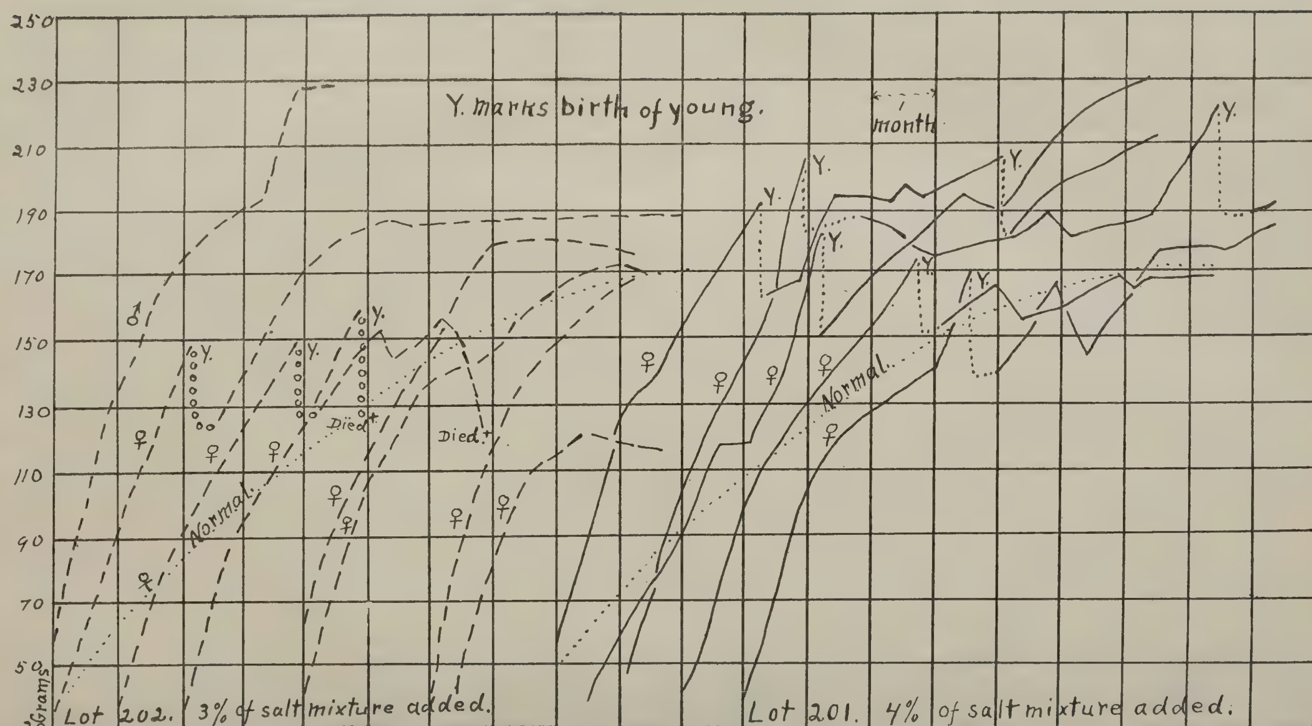


CHART IV. Shows the records of rats which received 3 per cent (Lot 202) and 4 per cent (Lot 201) respectively of the salt mixture (Table III) per 100 gm. of Ration 205. There is no evidence of improvement in these rats in the matter of reproduction or ability to rear their young, over those receiving smaller additions of the salt mixture (Chart III). Details regarding the histories of the young are given in Table II.

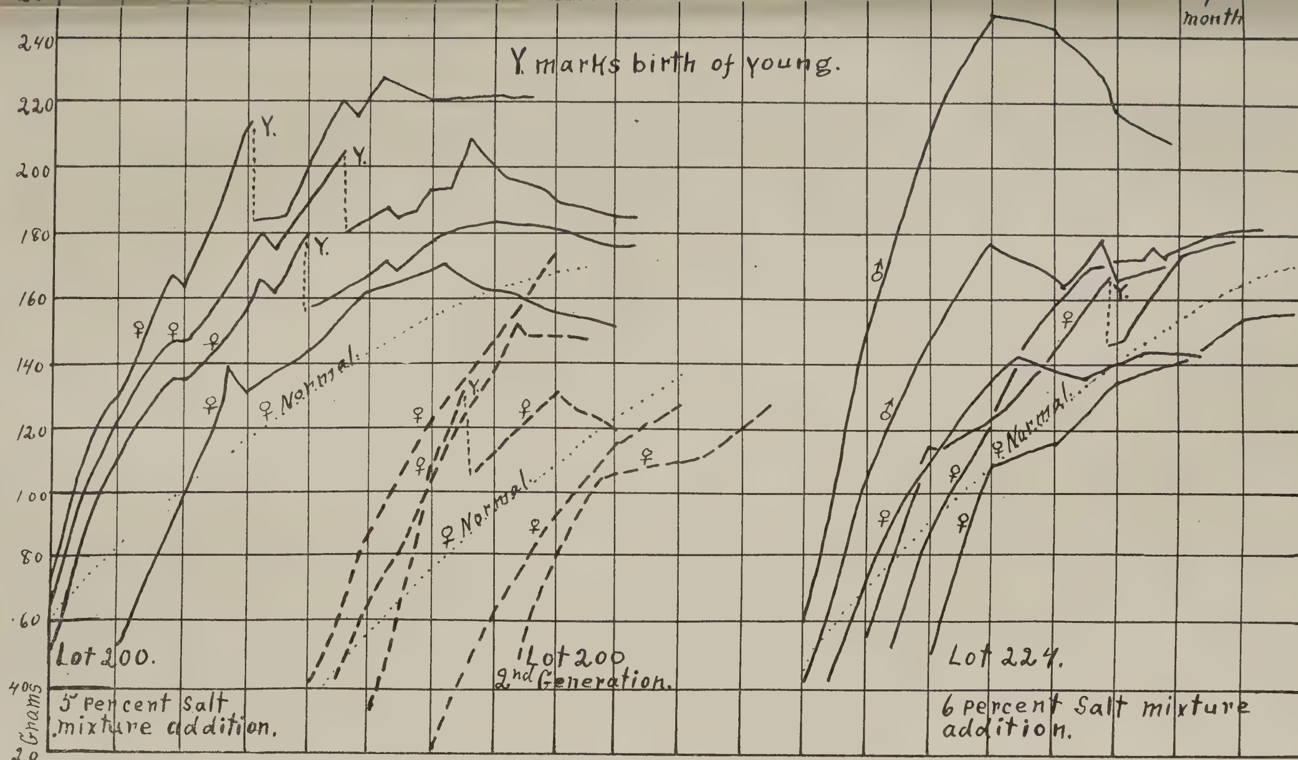
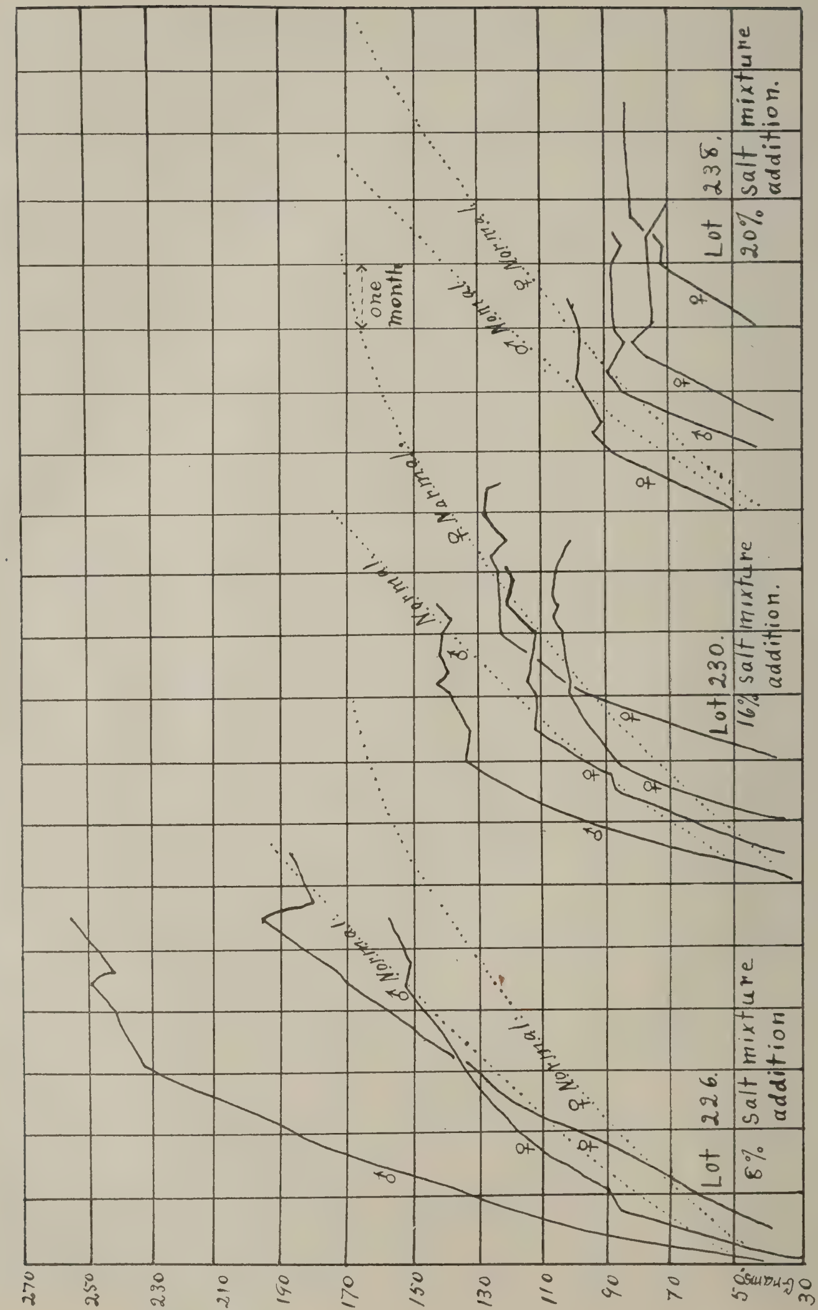


CHART V. Shows the records of Lot 200 which received 5 per cent addition of the salt mixture (Table III) to Ration 205 (Chart II). While only three litters of young were secured all but one individual of these were successfully weaned and grew well on the rations of their mothers. In no other lot was this degree of success attained in the rearing of the young. It is possibly of significance in this connection that the degree of alkalinity of this ration corresponds closely to that of a mixture of milk powder and dextrin which would supply the protein carried by this ration (see footnote to Table I). One female in this lot has never produced young.

In Lot 224, receiving 6 per cent of the salt mixture with Ration 205, normal growth but nearly complete failure of reproduction was observed. Unfortunately the one rat which produced young was not isolated and the young were eaten by her companions.



Lot 226. 6% Salt mixture addition
Lot 230. 16% Salt mixture addition.
Lot 238. 20% Salt mixture addition.
one month
♂ Normal
♀ Normal
grams
270
250
230
210
190
170
150
130
110
90
70
50
30

Chart VI. Lot 226, receiving Ration 205 with 8 per cent of the salt mixture addition grew well, but no young were secured.

Lot 230, receiving 16 per cent of the salt mixture grew normally during the first eight weeks, but at that point growth became suspended. They still appear to be in good condition. The rapid growth at first indicates that the animals were able to eat enough of the ration for growth, and suggests that either the high salt intake or the high alkalinity was responsible for the cessation of growth.

Lot 238, receiving 20 per cent of the salt mixture, increased in weight rapidly for a month, after which growth was completely suspended. We cannot say definitely whether it was the high base content or the high content of the organic acid radicals in the salt mixture which depressed the growth of the animals. This question will be further studied.

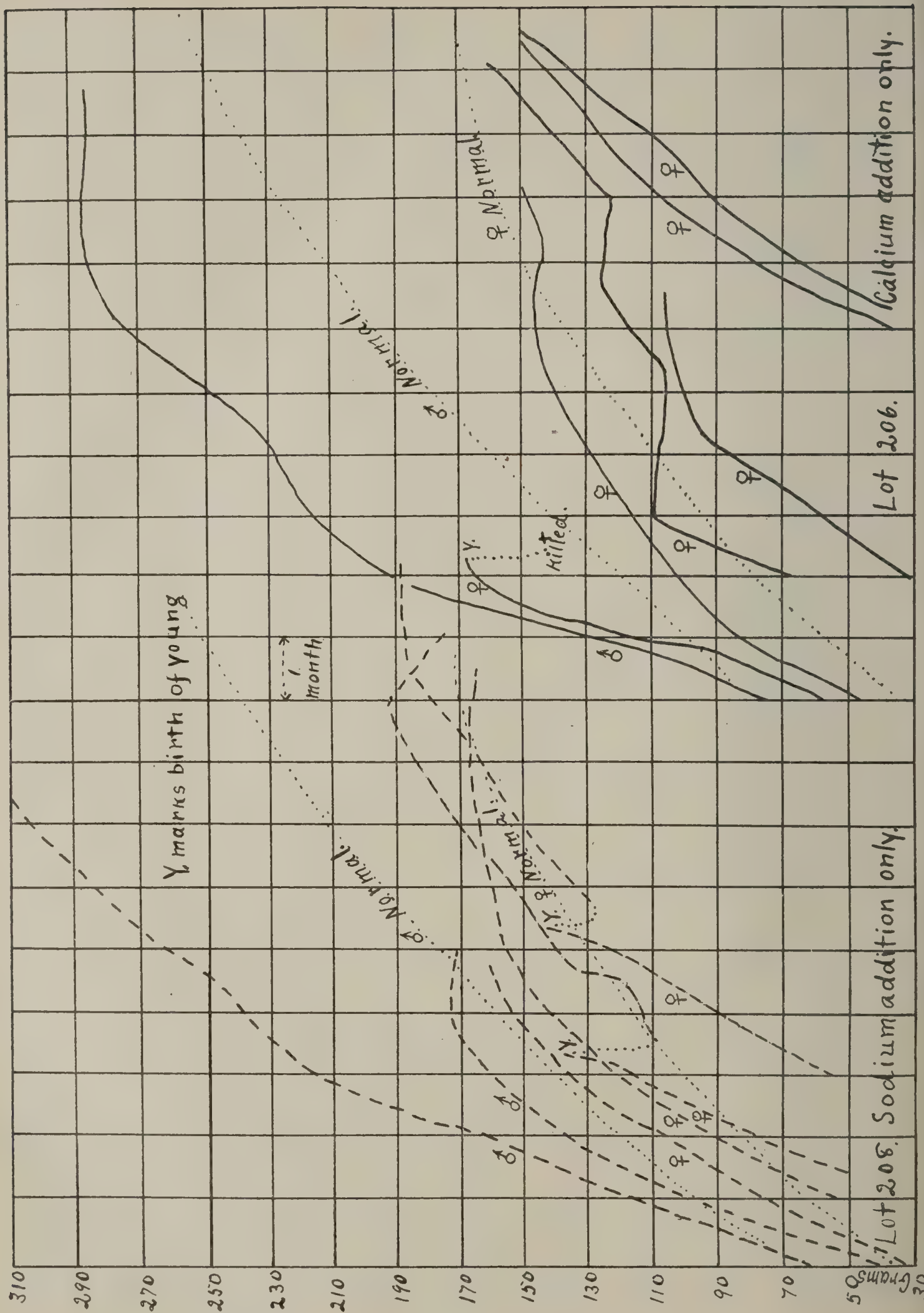

Calcium addition only.
Lot 206.
Sodium addition only.
Lot 208.
Y marks birth of young
1 month
♀ Normal
♂ Normal
♀ Normal
♂ Normal
Killed.
310
290
270
250
230
210
190
170
150
130
110
90
70
50
30
Grams

CHART VII. Shows the effect of adding a high content of a single base to Ration 205. In each case this addition was sufficient to produce a moderate alkalinity in the ration (see Table I).

Lot 208, receiving Ration 205 with sodium citrate as the only salt addition, made normal growth and produced two litters of young. In normal rats probably ten litters would have been produced in this period by four females. It is interesting that six of ten young lived to weaning time. No second litters have been secured on the ration.

Lot 206 received Ration 205 with calcium lactate as the only salt addition. Growth has been practically normal, but reproduction has been limited to the birth of a single litter. The mother ate her young and was killed.

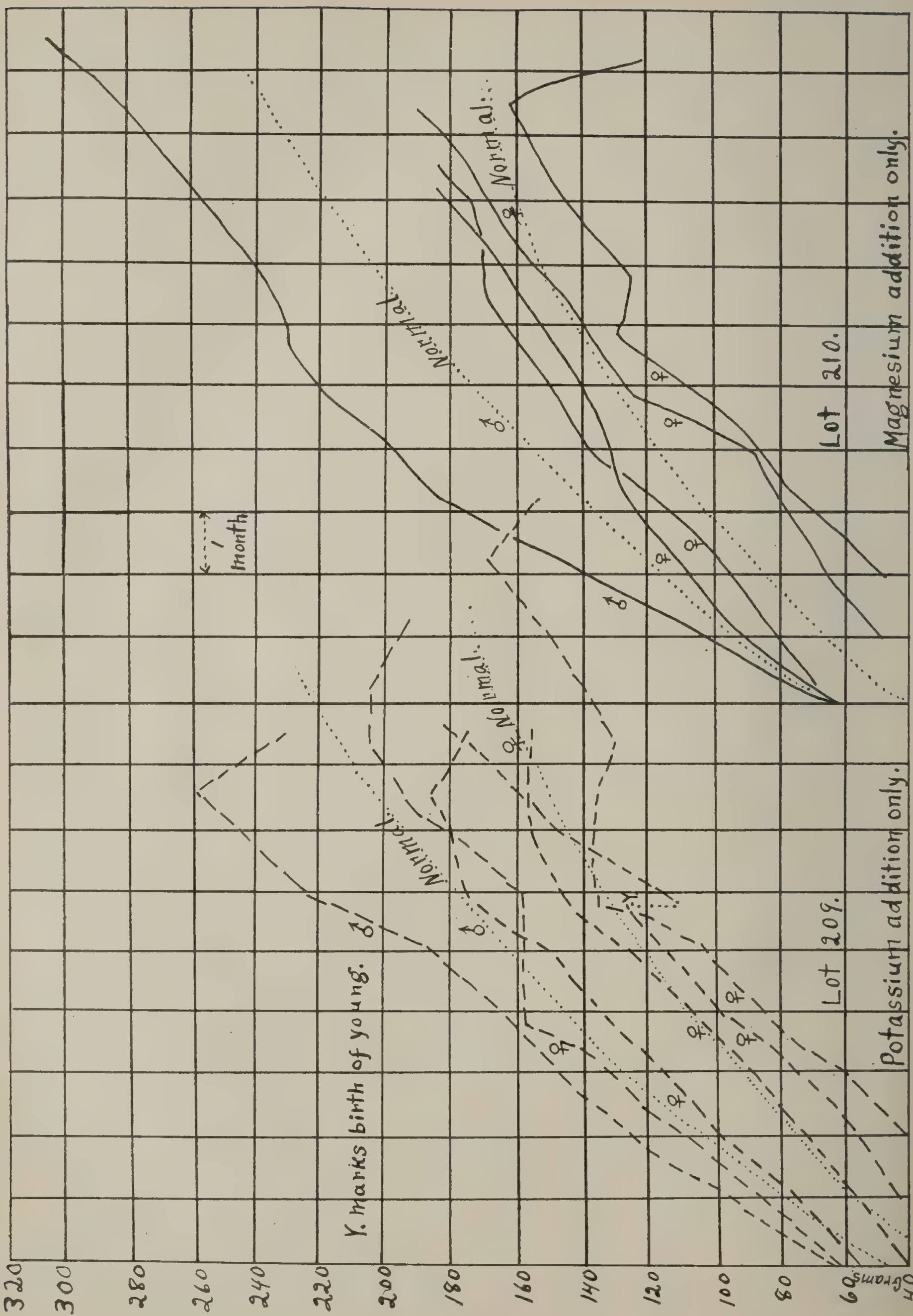

Lot 210.
Magnesium addition only.
Lot 209.
Potassium addition only.
Y. marks birth of young.
1 month
Normal
Grams
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40

CHART VIII. Shows the behavior of rats fed Ration 205 with the addition of a single base sufficient to produce a moderate alkalinity in the ration. Lot 209 received potassium citrate as the only salt addition. Growth was not interfered with, but with the exception of a single young one there was no reproduction, although some of these females are nearing the end of the period of sexual activity.

Lot 210 received magnesium citrate as the only salt addition. This high magnesium content has not interfered with growth, although the content of this element was so high as to produce a slight constant diarrheal condition. No young have been produced by this group.

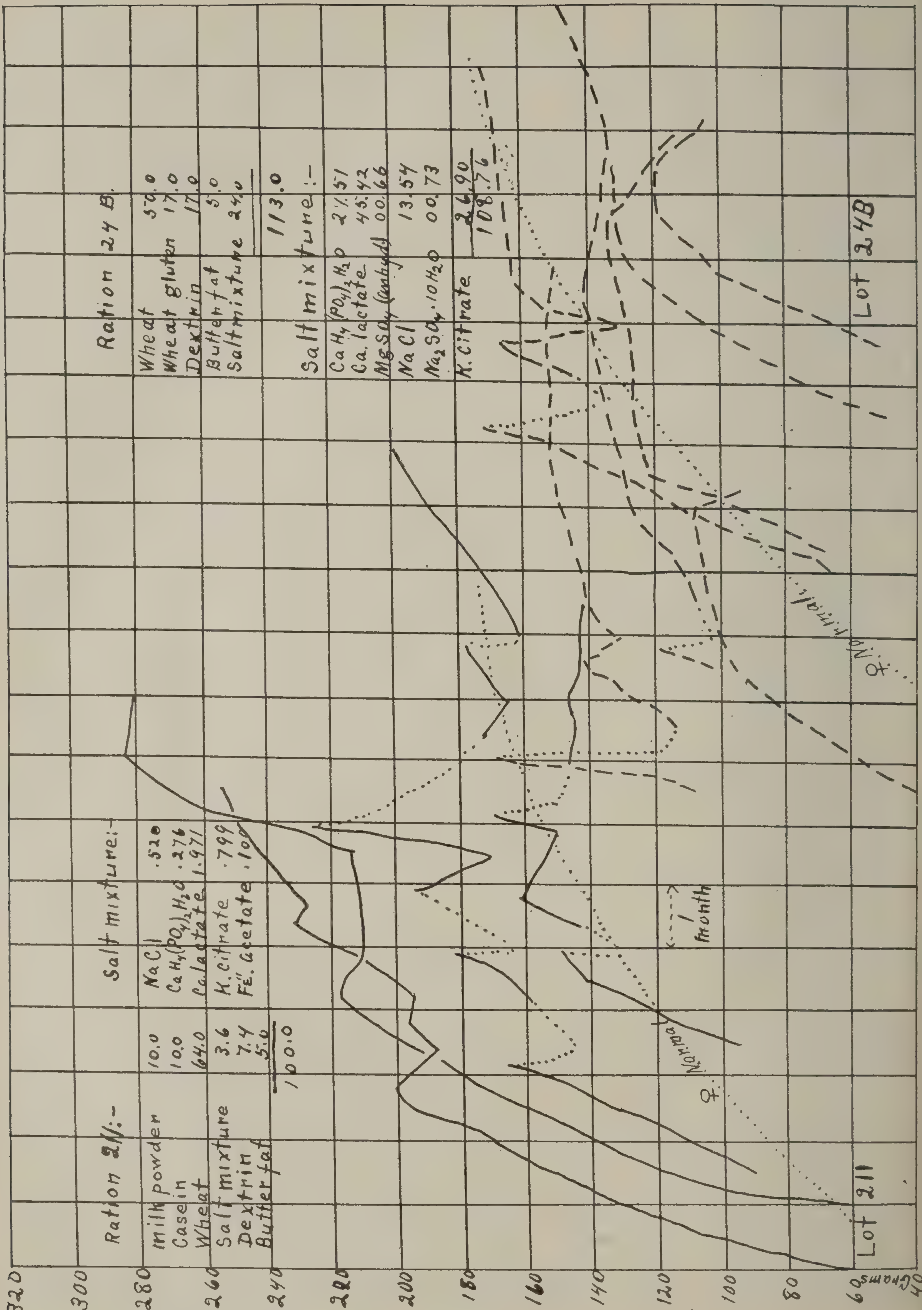

Ration 211:-
Milk powder 10.0
Casein 10.0
Wheat 64.0
Salt mixture 3.6
Dextrin 7.4
Butter fat 5.0
100.0
Salt mixture:-
NaCl .520
CaH4(PO4)2H2O .276
Ca lactate 1.971
K. citrate .799
Fe. acetate .10
Ration 24 B.
Wheat 50.0
Wheat gluten 17.0
Dextrin 17.0
Butter fat 5.0
Salt mixture 24.0
113.0
Salt mixture:-
CaH4(PO4)2H2O 21.51
Ca. lactate 45.42
MgSO4 (anhyd) 00.66
NaCl 13.54
Na2SO4.10H2O 00.73
K. citrate 26.90
108.76
Lot 211
Lot 24B
♀ Normal
♀ Normal
1 month
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
Grams

CHART IX. Lot 211. The records of these rats illustrate the marked improvement in repeating reproduction and rearing the young as compared with Lot 201 whose ration was closely similar with respect to its inorganic content, but contained no wheat. Lot 211 reproduced at frequent intervals and all young were properly nourished. The replacement of the wheat in this ration by an equivalent amount of protein and carbohydrate in the form of casein and dextrin (Lot 201) leads to failure of reproduction and proper nutrition of the offspring. Evidently some factor other than the mineral content was responsible for the inadequacy of the rations described in Charts I to VIII.

Lot 24B. Curves represented by broken lines. The total protein content of the ration was supplied by wheat. The addition of salts and butter fat corrected this ration so that the animals were considerably better than those in Lot 223 (Chart XI), but second litters of young could not be secured with this ration. These rats remain in fairly good condition for months after failure sets in on a ration of wheat, casein, salts, and dextrin, but without butter fat (Lot 223). The young from Lot 24B lived from eleven to twenty days, unless they were put on a better ration. It is highly probable that the inorganic content of this ration is excessive.

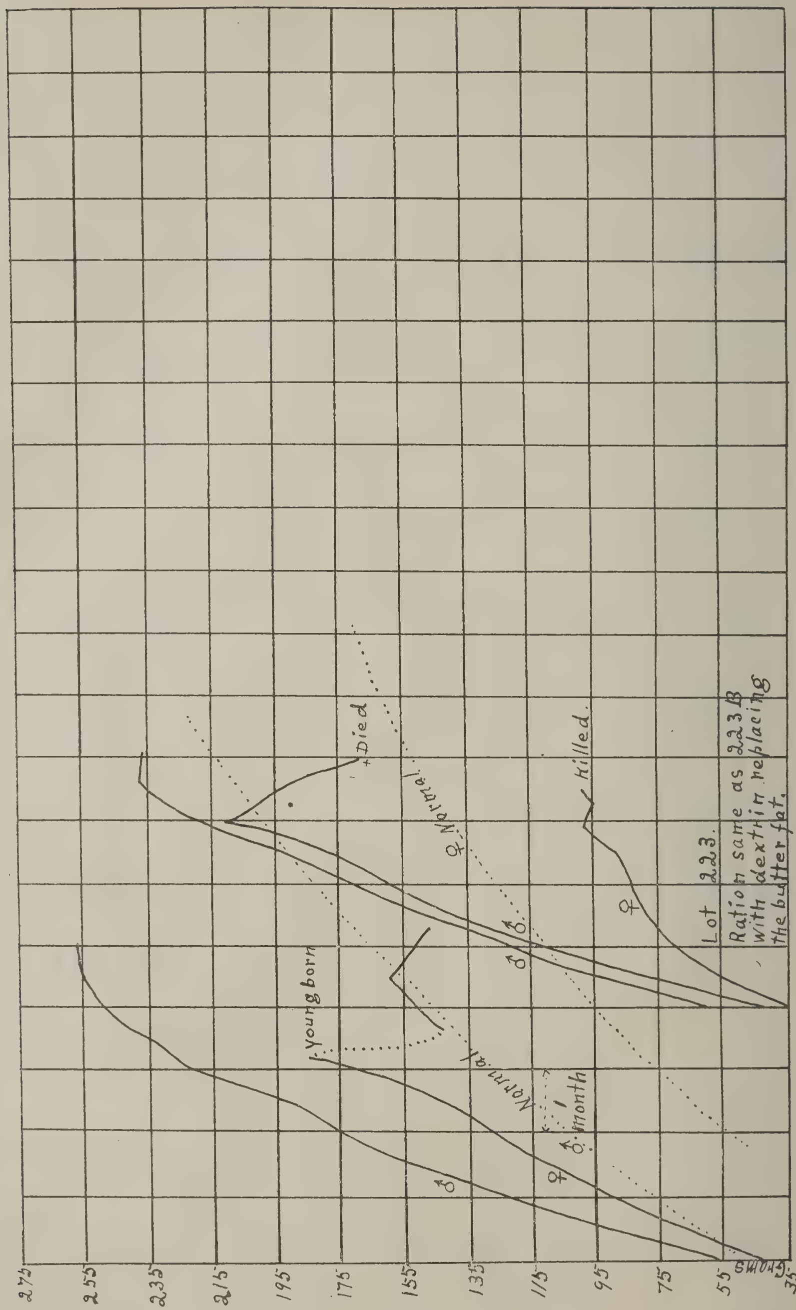

Lot 223.
Ration same as 223 B
with dextrin replacing
the butter fat.
Killed.
Died
♀ Normal.
Normal
Young born
1 month
♂
♀
Grams
275
255
235
215
195
175
155
135
115
95
75
55
35

Chart X. Lot 223. Here the correction of the protein deficiencies of wheat by the addition of casein and of the mineral content by salt additions induced normal growth for a time, and a single litter of young was secured. The animals presented a miserable appearance, however, after 4 months on this ration. With the addition of butter fat to this ration it becomes entirely adequate (Chart XI). When but a single factor in the ration is unfavorable, the animal is able to preserve itself through its factors of safety and may appear perfectly normal for a considerable period. This makes it very difficult to demonstrate the specific effects of the inorganic content of the ration on animals whose diet is in other respects fairly satisfactory (Charts I to VIII).

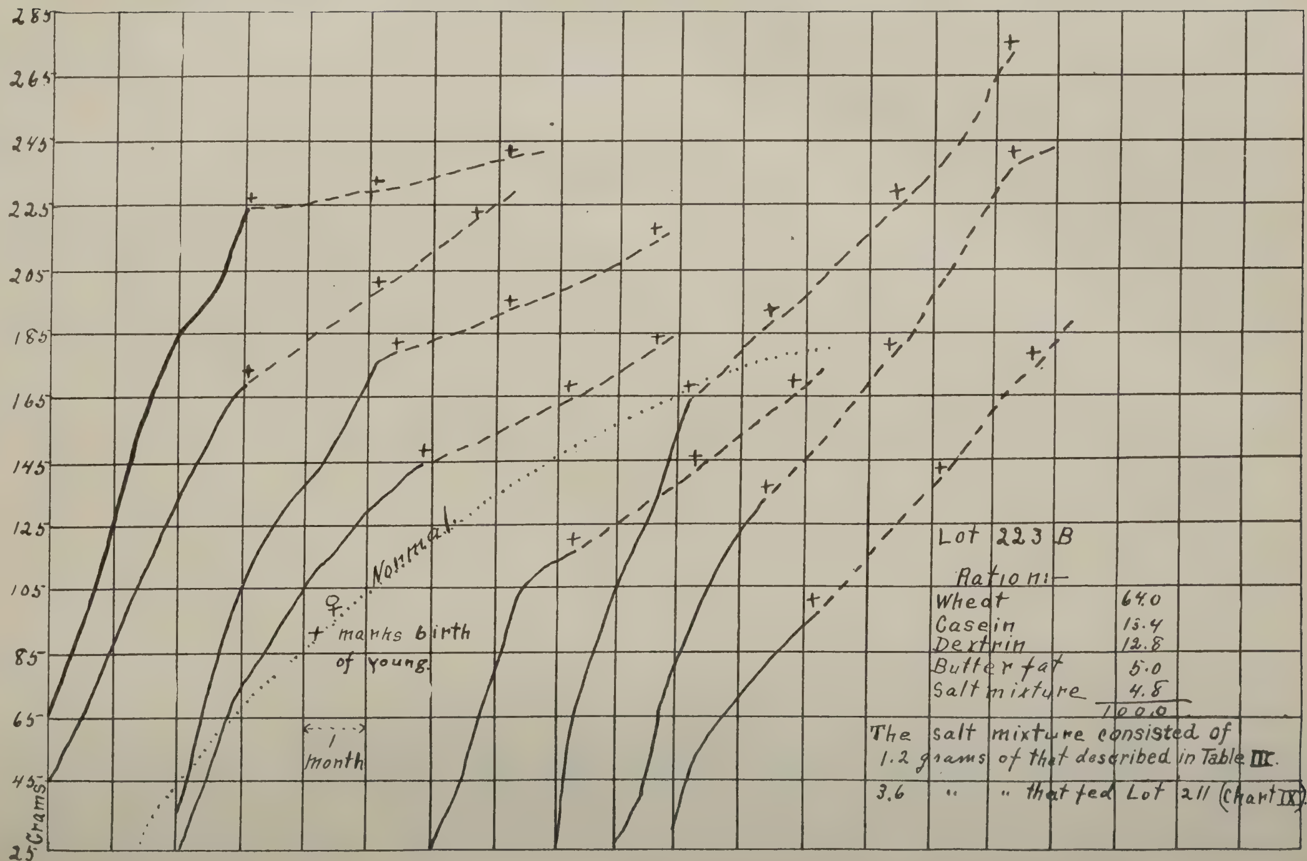

285
265
245
225
205
185
165
145
125
105
85
65
45
25
Grams
Normal
♀
+ marks birth of young.
1 month
Lot 223 B
Ration:—
Wheat 64.0
Casein 15.4
Dextrin 12.8
Butter fat 5.0
Salt mixture 4.8
100.0
The salt mixture consisted of
1.2 grams of that described in Table III.
3.6 " " that fed Lot 211 (Chart IX).

CHART XI. Lot 223B. These records illustrate in a striking manner the fact that by the addition of "pure" food substances, as protein and salts, wheat cannot be supplemented to produce a perfect ration as can the corn kernel. The unknown accessory article carried by certain fats must also be added to wheat before it becomes entirely adequate. Without salt additions we have not had success with rations of the type here employed. In Lot 223B the reproduction records show clearly the physiological sufficiency of this diet in a manner which could not be determined by observations on growth alone.

These females were not weighed during the period covered by the broken lines in the curves. The end of each curve shows the present weight of the mothers, and the crosses mark the birth of the litters.

NOTE ON THE BLACKENING OF THE LEAVES OF THE WILD INDIGO (BAPTISIA TINCTORIA) AND THE ISOLATION OF A NEW PHENOL, BAPTISOL.

By ERNEST D. CLARK.

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(Received for publication, May 17, 1915.)

Nature of the blackening of leaves.

There is nothing striking about the plant *Baptisia tinctoria* until it is bruised or frost-bitten; but then its rapid change from green to jet black makes it an interesting object. In fact, during colonial days a substitute for indigo was prepared from the plant; thus accounting for its popular name of "wild indigo." There are many other plants which are known to blacken upon injury, but few of our native species show this peculiarity in a more interesting way than this one does. In England and France the nature of this blackening process has been studied, and it is desirable to consider the bearing of these investigations on the present case.

During 1909 and 1910 Mirande,[1] Guignard,[2] and Maquenne with Demoussy[3] published the results of their work on blackening and cyanogenesis in leaves. They used plants like black mustard that produce a characteristic odor when frozen or treated with certain vapors; *Prunus* species giving hydrocyanic acid gas; and aucuba leaves, which blacken during narcosis. By the use of these different types of leaves as indicators of such changes the French authors all came to the same conclusion in regard to the phenomena of blackening, production of hydrocyanic acid, etc. They found that exposure to ultra-violet light, and to the vapors of certain substances, freezing with ethyl chloride, mechanical injury, and heat would all cause the typical change in color or production

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[1] N. Mirande: *Compt. rend. Acad. d. sc.*, cxlix, p. 140, 1909; cli, p. 481, 1910.

[2] L. Guignard: *ibid.*, cxlix, p. 91, 1909.

[3] L. Maquenne and Demoussy: *ibid.*, cxlix, p. 957, 1909.

of odor. However, if the green leaves were first plunged into boiling water they still remained green when treated in a way that caused blackening in normal unheated leaves. When leaves were dried quickly in a vacuum over sulphuric acid they did not blacken with vapors but did so at once when subsequently moistened with water.

All the evidence seems to indicate that these effects are controlled by enzymes of at least two different types. The first change seems to be the enzymic hydrolysis of some glucoside, like aucubain, with the production of glucose and a pigment later turning black. Some observers believe that oxygen and an oxidase are necessary for the production of blackening by the oxidation of a chromogen previously set free by an emulsin-like action on a glucoside. In other words, a hydrolytic enzyme first splits the glucoside in the leaves to give glucose and a chromogenic substance which, by the action of an oxidase, then gives the dark colored pigment. It is not certain that the blackening takes place in these two stages, but it seems likely. In the case of *Baptisia tinctoria* we know from the work of Emerson[4] that active oxidases are present in the leaves of this species.

It is certain that any agency which disturbs the normal relationships of the cells containing the enzyme and those containing the typical substrate will cause blackening, or the production of hydrocyanic acid, or whatever the typical reaction of that plant may be. This phenomenon is somewhat analogous to the plasmolysis of cells caused by subjecting them to the action of solutions of sugars, etc., having high osmotic activity. The changes in the cell preceding death of the protoplasm seem to cause the blackening, but it must be remembered that plunging the leaves into boiling water and also rapid dehydration prevent the blackening. It seems safer, then, to say that agents which tend to allow easy exchange of soluble material between cells (hence, greater permeability) will cause blackening, etc. In the normal living leaf this interchange between the cells containing enzyme and those containing substrate does not occur; but active vapors, cold, and mechanical injury alter the permeability and so encourage diffusion. Of course, the enzyme and substrate may be separated in some way from each other in different parts of the *same* normal cell, but the principle is the same.

In England, H. E. and E. F. Armstrong[5] studied the phenomena of cyanogenesis and blackening in leaves. By using leaves of the cherry laurel (*Prunus laurocerasus*) containing the glucoside prulaurasin, they investigated the effect of different vapors and inorganic salts in solution. They found that many vapors like toluene, chloroform, and esters, as well as certain salts, like cadmium iodide, potassium fluoride, etc., may all cause the formation of hydrogen cyanide, benzaldehyde, and glucose in the leaves of this plant. They detected the hydrogen cyanide with sodium picrate paper. In most cases the blackening first appeared in small dots

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[4] J. T. Emerson: *Bull. Torrey Botanical Club*, xxxi, p. 621, 1904.

[5] H. E. Armstrong and E. F. Armstrong: *Proc. Roy. Soc., Series B*, lxxxii, p. 588, 1909–10.

on the lower side of the leaves. Under the influence of the active substances the leaves absorbed considerable water when in contact with it. From their observations the Armstrongs concluded that the cell surfaces are coated with a protective sheath of water molecules which prevents the passage of substances having an affinity for water, but lets pass those active substances like esters, etc., having no attraction for water. In a later paper[6] by these same authors they record more experiments and develop this idea of the importance of the affinity of substances for water in determining their power to promote cellular activity. They also found, as did the French investigators, that there seems to be no relation between the chemical structure and the power to cause these changes in leaves. They believe that the main factors are "the degree of affinity of the substance for water and solubility relationships." According to Overton's well known theory, the plasma membranes contain lipoid material which permits ingress of lipoid solvents like ether and chloroform, but excludes many other substances not miscible with lipoids. However, modifications in this conception are necessary to explain the entrance of sugars, amino-acids, and salts known to be indispensable for the life of the cell, but which are not miscible with lipoids or soluble in their solvents.

The publications of the English and French investigators who worked with plants sensitive to the action of vapors, stimulated our interest in making similar studies upon *Baptisia tinctoria*, which is a common plant in sandy places, especially on the Atlantic Coast. This paper is a report of our experiments on the effects of various active substances in causing blackening of *Baptisia* leaves. In connection with these experiments we isolated and prepared derivatives of a new substance in the white efflorescence on the blackened leaves.

Experiments on the blackening of Baptisia leaves.

The fresh *Baptisia* leaves used in all our work were collected during the months of July and August in the vicinity of Cold Spring Harbor, Long Island, N. Y.[7] In many sandy places this plant is the only one growing in any quantity. We were able to prepare a considerable amount of this material for our various experiments.

[6] Armstrong and Armstrong: *Ann. Botany*, xxv, p. 507, 1911.

[7] During the summers of 1911 and 1912 this material was collected and prepared with the help of Dr. Ross A. Gortner, of the Carnegie Station for Experimental Evolution, Cold Spring Harbor, N. Y. Our thanks are due him and the Institution for this assistance.

We soon noted that the vapors of volatile organic substances caused the leaves of *Baptisia* to turn jet black. The rapidity of this change seemed to vary with the different substances used. The first sign of blackening appeared on the under side of the leaves in the form of black spots which quickly enlarged and spread in every direction, the upper surface being the last to blacken. This was probably due to the entrance of the vapors into the tissue of the leaf *first* through the stomata which were more numerous on the lower than on the upper leaf surface. The parts of the leaves and stems which were under the surface of water kept their original green color even after long exposure to the vapors had saturated the water with the vapors above it. It may be that the waxy covering of the leaves prevented the entrance of water containing the dissolved vapors, or else the water did not have enough dissolved oxygen to allow the formation of the black pigment to take place.

When masses of the *Baptisia* leaves were subjected to the action of vapors of chloroform or ether in large jars, the temperature began to rise at once from 25° to 28° and often reached 55° to 60° in an hour, when a thermometer was plunged into the center of the mass. These heat effects could hardly have been caused by microorganisms in view of the presence of antiseptics like chloroform, toluene, etc. Plunging the fresh leaves into boiling water did not cause blackening and it also prevented subsequent blackening by any agent. Many tests showed that there was very much less sugar in normal green leaves or those killed quickly by boiling water than in those which had been blackened in any manner. The observations recorded above are fundamental, and tend to show that the blackening is due to a somewhat complex enzymic process, in which oxygen and also some hydrolytic enzyme play a part. During the blackening the surface of the leaves became covered with a delicate white efflorescence of frost-like crystals. The latter part of this paper deals with the isolation and study of this crystalline material.

The relative effects of different vapors upon the *Baptisia* leaves were determined in the following way: Glass vials of uniform volume were fitted with tight corks. From a pipette, exactly 1 cc. of each liquid was carefully run into the bottom of each vial. A branch of the *Baptisia* plant containing three leaves was

then corked in the upright vial but not allowed to touch the liquid. Under uniform temperature conditions, the time required for the leaves to become jet black was noted. This could be determined with some accuracy, amounting to about one minute in fifteen. Several series of these experiments were made and they gave concordant results for each volatile substance used. Among the different active compounds there was the greatest variation in the time required for the leaves to become completely blackened. In order to see if there was any relation between chemical constitution, boiling points, or solubilities and activity when tested on *Baptisia* leaves, all of these data were condensed into one table which is given below. The time given that was required to blacken is the average of the results of several experiments.

TABLE I.

Rate of action of vapors on Baptisia leaves at 22° to 24°.

SUBSTANCE	FORMULA	TIME REQUIRED TO BLACKEN	BOILING POINT IN DEGREES	SOLUBILITY IN WATER* AT 22–24°	SOLUBILITY IN ETHER
Acetone	$CH_3-CO-CH_3$	10 min.	56.5	Infinite	Infinite.
Acetic acid	$CH_3-COO\ H$	Bleached	118	"	"
Ethyl acetate	$CH_3-COO\cdot C_2H_5$	17 min.	77	6%	"
Ethyl butyrate	$CH_3-COO\cdot C_4H_9$	11 min.	121	Slight	Soluble.
Carbon bisulphide	$C\ S_2$	25 min.	46	0.22%	Infinite.
Benzene	$C_6\ H_6$	18 min.	80	0.07%	"
Toluene	$C_6H_5-C\ H_3$	22 min.	111	Slight	"
Hexane (*n*)	C_6H_{14}	Inactive	69	Slight	"
Ethyl ether	$C_2H_5-O-C_2H_5$	22 min.	35	8.1%	"
Ethyl alcohol	$C_2H_5-O\ H$	33 min.	78	Infinite	"
Pyridine	C_5H_5N	Inactive	115	"	"
Chloroform	$C\ H\ Cl_3$	16 min.	62	0.63%	"

* Solubilities are taken from *Chemical Annual*, 2d edition, 1909.

From this table it seems that the most active substances are not the volatile antiseptics like toluene, chloroform, ether, etc., but are substances of the ester type, and also acetone. Vapors of inorganic acids and ammonia produced non-typical brown colorations and rapid destruction of tissue. As reported by the Armstrongs and the French investigators already mentioned, there appears to be no definite relation between the chemical structure or physical properties like solubilities and boiling points of the substances and their rate of action on the leaf. In general, the more chemically inert substances like esters were the most

efficient in producing the typical effects. It is interesting to note that acetone is the most active in causing the blackening, yet it is known to be less of a lipoid solvent than ether or chloroform. This fact is not in harmony with Overton's theory unless we assume that the lipoids of this plant are different from many others.

Isolation of a new substance from the efflorescence.

The fresh green *Baptisia* leaves were loosely packed in large battery jars containing about 25 cc. of ether or chloroform in a small beaker. In less than half an hour the whole mass of leaves became jet black, this change being accompanied by a distinct rise in temperature in the interior of the mass. After standing over night with the vapors, the leaves were found to be covered with a white efflorescence. These leaves were then taken out of the jars and spread out to dry in the air. The dry material could be kept indefinitely in this state.

Alcohol easily dissolved the efflorescence on the dry leaves, but when this solvent was used on a large scale it was found impossible to obtain a pure preparation because of the amount of chlorophyl dissolved. The most satisfactory way to prepare the pure substance was as follows:

The dry leaves were placed in a 0.5 per cent solution of sodium hydroxide, and the mixture was heated nearly to the boiling point; the leaves were then strained off through cheese-cloth, and the yellowish solution filtered through paper. Upon neutralization with very dilute acetic acid solution, a flocculent precipitate of crude material came down. This product was again dissolved in very dilute sodium hydroxide solution, which was then filtered and reprecipitated with a few drops of acetic acid. A lighter colored precipitate was thus obtained, and, after washing, was allowed to dry on the filter paper. The dry filters containing the crude substance were then extracted with hot 95 per cent alcohol. The yellowish alcoholic solutions were boiled with bone-black and, after filtration, these hot solutions were diluted with hot water just to the point (about 30 per cent alcohol) where a slight cloudiness appeared. After cooling and standing in the ice box over night a white powdery preparation was obtained having a melting point of 213°. Repeated crystallizations from benzene, acetic acid, chloroform, etc., caused no change in this melting point.

When pure, the new substance is a felted mass of fine white needles melting sharply at 213° (corrected). In the manner just

described, several preparations of the pure substance were made. All agreed in having a melting point of 212–13°. The total amount of the pure preparations obtained from the leaves was small. In all, 3.5 kg. of air-dry leaves gave a total weight of pure substance of only 8 grams, so the amount of that substance in the dry leaves was about 0.2 per cent. It should be noted that the fresh green leaves yielded none of the new substance when treated with warm alkaline solutions in the same manner as the blackened leaves. Whether this product is formed in the leaves by an enzymic action accelerated by the active vapors, or whether it merely crystallizes on the leaf surface because of changes in its solute is impossible to say. However, in certain other plants, it is known that enzymes hydrolyze complex glucosides to produce phenols under the influence of active vapors.

Properties of the new substance.

This substance is insoluble in water, but dissolves in glacial acetic acid, concentrated hydrochloric acid solution, benzene, alcohol, chloroform, etc. It readily goes into solution with fixed alkalies to give a yellow color, and under such conditions is precipitated unchanged by very dilute acids or carbon dioxide. Heated with metallic sodium or soda-lime it gave no indications of the presence of nitrogen, sulphur, or the halogens. With very dilute alcoholic solutions of ferric chloride an evanescent cherry-red color appeared, but faded almost instantly to a brownish shade. When tested for phenol coloration by Alsberg and Black's[8] test, the chloroform layer became pink in color, both with and without the addition of a trace of hydrogen peroxide. With Millon's reagent it gave a typical deep red color. This and other evidence indicates that the new substance is a phenol. In carbon tetrachloride solution there was no absorption of bromine. A hot alkaline aqueous solution of the phenol is readily oxidized by potassium permanganate. Zinc dust is without effect on it, either in a solution with sodium hydroxide or glacial acetic acid. This phenol does not reduce alkaline silver or copper solutions nor does it combine with phenyl hydrazine or hydroxylamine.

[8] C. L. Alsberg and O. F. Black: *U. S. Department of Agriculture, Bureau of Plant Industry, Bull. 270*, p. 42, 1913.

Tests with alcoholic potassium acetate solution for flavone derivatives containing hydroxyl groups in the ortho position (according to Perkin and Wilson)[9] were negative. Perkin and Hummel used alcoholic lead acetate solution as a test for the catechol complex in flavones, and this, too, was negative with the new substance. Fusing it with phthalic anhydride and a few drops of sulphuric acid at 160° and rendering alkaline, produced no fluorescent substance, so the hydroxyl groups are apparently not in the meta position. It seems likely, then, that the new phenol is a complex one, probably not of the flavone series, and is not identical with any known substance. Later this will become more evident, when the analytical results are shown.

Composition of the new phenol.

Three different preparations were used for analysis. The first one (I) was impure and melted at 210–11°, but the second and third (II and III) were dry and pure, melting at 213° (corrected). Heating the new substance at various temperatures failed to show any loss of weight, due to loss of water or alcohol of crystallization. The combustions for determining the percentage of carbon and hydrogen were made according to the method of Dennstedt, but it should be noted that this phenol and its derivatives do not burn smoothly.

Percentage of carbon and hydrogen in the new phenol.

SAMPLE	WEIGHT OF SUBSTANCE	WEIGHT OF CO_2	WEIGHT OF H_2O	C	H
	gm.	*gm.*	*gm.*	*per cent*	*per cent*
Preparation I (impure)	0.1200	0.2826	0.0534	64.21	4.98
Preparation II (pure)..	0.1196		0.0484		4.53
" " " ..	0.1442	0.3486	0.0610	65.92	4.73
" III " ..	0.0906	0.2200	0.0362	66.20	4.47
" " " ..	0.0954	0.2307	0.0373	65.98	4.38
$C_{15}H_{12}O_5$ requires........				66.16	4.41

Next, the molecular weight was determined by the freezing point method. The use of benzene as a solvent for this purpose

[9] A. G. Perkin and C. R. Wilson: *Tr. Chem. Soc.*, lxxxiii, p. 129, 1903.

proved to be impossible because the substance crystallized out above the freezing point of this solvent. Acetic acid was finally used as a solvent and the results of the determinations are tabulated below.

Molecular weight of the substance by the cryoscopic method.

SAMPLE	WEIGHT OF SUBSTANCE	WEIGHT OF SOLVENT	K OF SOLVENT	Δ OBSERVED	M FOUND
	gm.	*gm.*			
Preparation III (pure) . .	0.1956	63.0	39	0.135°	90
" " " . .	0.1074	26.25	39	0.171°	93
$C_{15}H_{12}O_5$ requires.... ...					**272**

Evidently we have here a case of an abnormal freezing point, since the value found for the molecular weight is one-third that required by theory. It is apparent from other observations that the substance is much more likely to have a molecular weight of 272 than 90.

Number of methoxyl groups in the substance.

In order to find the number of methoxyl groups present, the necessary apparatus was arranged and the determinations were made according to Perkin's[10] modification of Zeisel's method. Some experience with this method is required before one can secure satisfactory results. As soon as nearly theoretical figures were obtained for methoxyl groups in pure vanillin, the determination was made upon the pure phenol, care being taken not to heat the reaction flask above 128–130° (boiling point of hydriodic acid solution used), nor to pass the carbon dioxide through the solution too rapidly. The determinations agreed very well and showed that the new phenol has but one methoxyl group. Below are given the data of a typical analysis.

[10] W. H. Perkin: *ibid.*, lxxxiii, p. 1367, 1903.

Determination of methoxyl groups.

SAMPLE	WEIGHT OF SUBSTANCE	WEIGHT OF Ag I	WEIGHT OF CH_3O FOUND FROM Ag I	CH_3O FOUND	CH_3O CALCULATED
	gm.	*gm.*	*gm.*	*per cent*	*per cent*
Preparation III (pure)..	0.1260	0.1009	0.0134	10.7	
$C_{14}H_9O_4OCH_3$ requires...					**11.36**

Preparation of certain derivatives of the new phenol-triacetyl derivative.

The properties of the new substance and certain of its color tests indicated that it was a phenol. An acetyl derivative was prepared as follows: 5 grams of freshly fused sodium acetate were mixed with 2 grams of the phenol, 20 grams of acetic anhydride added, and the mixture was boiled on an electric hot plate for two hours. Upon cooling, the contents of the flask nearly solidified into a mass of long hair-like white crystals in rosettes. After shaking with water until the excess of acetic anhydride had been removed, the acetyl derivative appeared in the form of white, needle-like crystals, which were insoluble in water but crystallized readily from glacial acetic acid. This new substance was boiled with acid-washed bone-black in acetic acid solution until a pure white product was obtained. The pure recrystallized triacetyl derivative melted at 189° (corrected). The pure dry substance was then analyzed for the percentage of carbon and hydrogen.

Percentage of carbon and hydrogen in the acetyl derivative.

SAMPLE	WEIGHT OF SUBSTANCE	WEIGHT OF CO_2	WEIGHT OF H_2O	C	H
	gm.	*gm.*	*gm.*	*per cent*	*per cent*
Preparation II (pure)...	0.1020	0.2362	0.0435	63.15	4.77
" " " ...	0.1025	0.2380	0.0417	63.33	4.56
$C_{15}H_9O_5\cdot(CO\cdot CH_3)_3$ or $C_{21}H_{18}O_8$ requires............				**63.32**	**4.52**

Analysis showed that a triacetyl derivative was produced by the action on the phenol of an excess of acetic anhydride. The determinations of the molecular weight of this new acetyl substance in acetic acid solution gave very irregular values which were only a fraction of the 398 required by the formula $C_{21}H_{18}O_8$.

However, all other data show the more complex formula to be correct and consequently the new substance contains three hydroxyl groups.

Some of the pure triacetyl derivative was saponified by boiling with sodium hydroxide solution. The mixture was acidified with phosphoric acid and distilled into a known amount of standard alkali, the excess being titrated.

Number of acetyl groups in the acetyl derivative.

SAMPLE	WEIGHT OF SUBSTANCE	AMOUNT OF $\frac{N}{10}$ NaOH	AMOUNT OF CH_3COOH FOUND	CH_3COOH FOUND	CH_3COOH CALCULATED
	gm.	*cc.*	*gm.*	*per cent*	*per cent*
Preparation II (pure)...	0.1331	10.20	0.612	45.98	
$C_{15}H_9O_5 \cdot (CO \cdot CH_3)_3$ requires...					45.33

Here again the presence of three hydroxyl groups in the original phenol is shown by the analysis.

Tribenzoyl derivative.

A small amount of the pure phenol was dissolved in pyridine, an excess of benzoyl chloride added, and the mixture heated on an electric plate for an hour. After cooling, the reaction mixture was shaken with dilute solutions of sulphuric acid and sodium carbonate until only a small yellowish cake remained. This cake was not soluble in 95 per cent alcohol, but dissolved very easily in benzene and ethyl acetate. From hot aqueous acetone this benzoyl derivative crystallized in silvery spangles whose melting point was always found to be 183° (corrected). A large scale preparation was made and crystallized from a mixture of acetone and water for purposes of analysis.

The benzoyl derivative was also prepared by dissolving the pure substance in 20 per cent sodium hydroxide solution, and to this was added benzoyl chloride in small portions. After standing for a time, the mass of foreign material was removed, leaving a solid cake of material which melted at 181° after repeated crystallizations from dilute aqueous acetone solutions. This preparation and others made in a similar way did not seem pure nor did they melt at 183° like those prepared by the use of pyridine.

Percentage of carbon and hydrogen in the benzoyl derivative.

SAMPLE	WEIGHT OF SUBSTANCE	WEIGHT OF CO_2	WEIGHT OF H_2O	C	H
	gm.	*gm.*	*gm.*	*per cent*	*per cent*
Preparation I.	0.0835	0.2257	0.0316	73.72	4.23
" I.	0.0910	0.2451	0.0352	73.45	4.33
$C_{15}H_9O_5 \cdot (C_6H_5 \cdot CO)_3$ or $C_{36}H_{24}O_8$ requires				**73.97**	**4.19**

There seems little doubt, then, that the excess of benzoyl chloride produced a tribenzoyl derivative.

A reduction product of the new phenol.

After the determination of methoxyl radicles in the original phenol, large yellow needles of an unknown substance crystallized from the concentrated hydriodic acid solution when it cooled. This substance was recrystallized from alcohol and finally obtained, in the form of clusters of snow-white needles melting above 260°. Some of the phenol itself was then placed in a bomb-tube with an excess of hydriodic acid solution (sp. gr. 1.70) and heated for three hours at 130°. When the tube was cold, it was opened and the contents were poured into water to precipitate the organic substance. After complete purification with alcohol as a solvent, we obtained a small quantity of white needles melting above 260°. This reduction product dissolved in alkalies with a yellow coloration and contained no double bond or iodine in its molecule. The purest preparation was analyzed for carbon and hydrogen. It seemed to be an unknown reduction product, but the amount of substance analyzed was too small to give figures of any value.

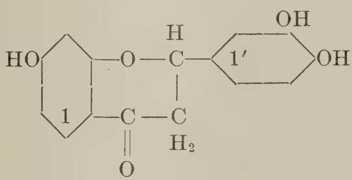
Percentage of carbon and hydrogen in the reduction product of baptisol.

WEIGHT OF SUBSTANCE	WEIGHT OF CO_2	WEIGHT OF H_2O	C	H
gm.	*gm.*	*gm.*	*per cent*	*per cent*
0.0183	0.0397	0.0104	59.17	6.36
$C_{15}H_{12}O_5$ (original substance) requires			**66.16**	**4.41**

Occurrence of substances similar to the new phenol.

In 1897 Gorter[11] investigated the constituents of the root of *Baptisia tinctoria,* which was formerly used in medicine. The raw material for his work was the residue left after evaporating the 60 per cent alcoholic extract of the root. He isolated the glucoside, baptisin, having the formula $C_{26}H_{32}O_{14}$ and melting at 240°. Upon hydrolysis of the baptisin with a dilute solution of sulphuric acid, one molecule of a new phenol, baptigenin, was produced, and two molecules of rhamnose. The molecular weight of baptisin as determined by freezing point depression in acetic acid was only 190, which is one-third of the theoretical value for $C_{26}H_{32}O_{14}$. The baptigenin crystallized from dilute alcohol in fine white needles melting above 250°. From analytical data and the preparation of derivatives it appeared that baptigenin has the formula $C_{14}H_{12}O_6$, with three hydroxyl groups but no other reactive groups that could be identified. It did not give any color with ferric chloride or Millon's reagent. Gorter was unable to show the constitution of any of the substances he isolated.

Power and Salway[12] isolated a new phenol from red clover flowers, which they called pratensol. This substance had the formula $C_{17}H_9O_2 \cdot (OH)_3$, melted at 210°, and gave a greenish black color with ferric chloride solution. The triacetyl derivative was prepared and found to melt at 189°. Perkin and Hummel[13] isolated a new phenol, butin, from the flowers of *Butea frondosa.* This substance has the formula $C_{15}H_{12}O_5$. Butin also has three hydroxyl groups, but differs from the new substance here reported in having no methoxyl group. Butin itself occurs in the form of small colorless needles melting at 224°, while its triacetyl derivative has a melting point of 123° and tribenzoyl butin melts at 155°. Upon fusion with potassium hydroxide butin yielded protocatechuic acid and resorcin. From this and other evidence Perkin and Hummel concluded that butin was 3, 3′, 4′-trioxyflavanon:



This assumption was proven to be correct in 1912 when Göschke and Tambor[14] synthesized butin and demonstrated that it has the above

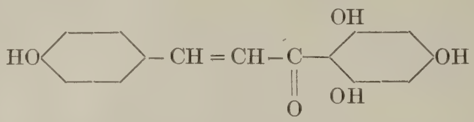
[11] K. Gorter: *Über die Bestandteile der Wurzel von Baptisia tinctoria. Dissertation,* Marburg, 1897. See also, Gorter: *Arch. d. Pharm.,* ccxxxv, pp. 301–332, 1897.

[12] F. B. Power and A. H. Salway: *Tr. Chem. Soc.,* xcvii, pp. 231–254, 1910.

[13] A. G. Perkin and J. J. Hummel: *ibid.,* lxxxv, pp. 1459–1472, 1904.

[14] A. Göschke and J. Tambor: *Ber. d. deutsch. chem. Gesellsch.,* xlv, pp. 186–188, 1912.

constitution. Finally, in the literature there is a description of another substance having the formula $C_{15}H_{12}O_5$ and known as naringenin. Will[15] prepared it by hydrolyzing the glucoside naringin isolated from the leaves and fruit of *Citrus decumana.* Naringenin occurs in the form of white platelets melting at 248° and giving a brownish red coloration with ferric chloride solution. Tutin[16] showed that naringenin had the following constitution:



Upon boiling naringenin with potassium hydroxide solution, phloroglucin and *p*-cumaric acid were produced. Our new phenol showed none of the reactions characteristic of naringenin, butin, or any of the flavone series. From this short survey of known phenols of the general formula $C_{15}H_{12}O_5$, we see that it is not possible to identify the new substance from the description of any other compound in the literature. Therefore, we propose to name it *baptisol,* since we first isolated it from blackened leaves of *Baptisia tinctoria* and first described its properties.

Constitution of baptisol.

From the results of the analyses of baptisol and its derivatives we may conclude that it contains one methoxyl and three hydroxyl groups. From the chemical and physical properties already described it is evident that baptisol is not identical with butin or naringenin, known substances having the formula $C_{15}H_{12}O_5$. Boiling baptisol with solutions of sodium hydroxide and fusion with caustic alkali did not yield protocatechuic acid and resorcin like butin, nor *p*-cumaric acid and phloroglucin like naringenin. Alkaline fusions of baptisol gave small amounts of a brownish oily substance having an odor of creosote. Some baptisol was dissolved in dilute sodium hydroxide solution and boiled with the addition of potassium permanganate until a pink color remained in the solution. The oxidation product was obtained in the usual way from the reaction mixture and found to have a pleasant odor like piperonal (heliotropin). After purification, it melted at 173°, gave a typical Millon test, and could not be

[15] W. Will: *Ber. d. deutsch. chem. Gesellsch.*, xviii, p. 1322, 1885; xx, p. 294, 1887.

[16] F. Tutin: *Jour. Chem. Soc.*, xcvii, p. 2057, 1910.

identified with any similar products obtained from butin and naringenin.

In summarizing the properties of baptisol we find that it apparently has no unsaturated bonds, no groups reacting with hydroxylamine or phenyl hydrazine, no groups capable of oxidation or reduction to form colored products, and none of the properties of flavones as a class. Baptisol has three hydroxyl groups and one methoxyl group, but no others that could be identified. Heating with hydriodic acid in a bomb-tube gave a white crystalline reduction product of greater hydrogen content than baptisol. We know that baptisol is not identical with butin or naringenin, but, unfortunately, owing to the small amount of material available we were unable to obtain data enough to enable us to suggest a probable structure for the new phenol.

SUMMARY.

1. The ordinary volatile antiseptics like chloroform, toluene, and ether were not nearly as active in causing the blackening of *Baptisia* leaves as were substances of the ester type and acetone. No relation could be demonstrated between the structure or physical properties of the active substances and their rate of action on the leaf. In general, the more chemically inert substances like esters were the most efficient in producing the typical effects.

2. The phenomena of blackening seem to be produced by any agency, physical, chemical, or mechanical, that disturbs the normal relationships of the cells containing certain enzymes and those containing the substrate; *i.e.*, altered permeability and active diffusion of cell constituents. Apparently, first a hydrolytic enzyme decomposes a glucoside, and then an oxidase acts to produce the dark pigment.

3. A new phenol, *baptisol*, was isolated from the efflorescence on the dried leaves of *Baptisia* blackened by vapors of chloroform, etc. Baptisol has the formula $C_{15}H_{12}O_5$ and melts at 213° (corrected).

4. *Triacetyl baptisol*, $C_{15}H_9O_5 \cdot (CO \cdot CH_3)_3$ was prepared and found to melt at 189° (corrected). *Tribenzoyl baptisol*, $C_{15}H_9O_5 \cdot (C_6H_5 \cdot CO)_3$ of melting point 183° (corrected) was also prepared. The formation of these compounds indicates the presence of three hydroxyl groups in baptisol.

5. It also contains one methoxyl group. We were unable to identify any other groups, and our present knowledge of the structure of baptisol may be indicated by the formula which follows:

$$C_{14}H_6O\ (OCH_3)\ (OH)_3$$

6. Baptisol is certainly not identical with either of the two phenols $C_{15}H_{12}O_5$, descriptions of which are to be found in the literature. One is butin from the flowers of *Butea frondosa*, and the other is naringenin from *Citrus decumana*, both substances having physical and chemical properties quite different from those of baptisol.

ON THE PRESENCE OF CHOLINE IN THE SHOOTS OF ARALIA CORDATA.

By K. MIYAKE.

(From the Chemical Laboratory, College of Agriculture, Tohoku Imperial University, Sapporo, Japan.)

(Received for publication, May 24, 1915.)

Since a search of the literature concerning *Aralia cordata* failed to reveal any results of special investigations on the nitrogenous constituents of its shoots, a study of the nature of its nuclein bases was undertaken, which resulted in proving the presence of guanine and xanthine.[1] A further study of the nitrogenous constituents has resulted in establishing the presence of choline in the juice of *Aralia* shoots. The method of investigation is as follows.

15 kg. of the fresh shoots were cut into small pieces, extracted with hot water, and the extract was treated with basic lead acetate. The filtrate from the lead compound was decomposed with hydrogen sulphide. The lead sulphide formed was filtered off, the filtrate being then neutralized with ammonia, and evaporated down to a liter on a water bath. After the addition of sulphuric acid until its content reached 5 per cent, a sufficient amount of phosphotungstic acid was added and the mixture well shaken. After twenty-four hours' standing, the precipitate formed was filtered off, thoroughly washed with 5 per cent sulphuric acid, and decomposed by baryta in the usual manner. The filtrate from the barium phosphotungstate was freed from baryta by carbon dioxide and evaporated again to a small volume. After being neutralized with nitric acid, concentrated silver nitrate solution was added, and the precipitate formed was collected on the filter and tested for the nuclein bases. To the filtrate from the silver compounds an excess of silver nitrate and baryta water was added. The brown precipitate formed was then filtered off, and the filtrate, freed from silver and baryta by hydrochloric and sulphuric acids, was again evaporated to a small volume. After sulphuric acid had been added until its content reached 5 per cent, a sufficient amount of phosphotungstic acid again was added, the mixture well shaken, and left untouched for

[1] K. Miyake: this *Journal*, xxi, p. 507, 1915.

about twenty-four hours. The precipitate formed was collected on the filter and washed with 5 per cent sulphuric acid. From this precipitate a solution which contains free bases was prepared according to the ordinary manner. After being neutralized with hydrochloric acid, it was evaporated to a syrup and dried in a desiccator for about one week. The dried syrup was then extracted many times with absolute alcohol. The extract was evaporated and the residue was dissolved in water. To this solution an excess of mercuric chloride was added and the mixture was allowed to stand for about forty-eight hours. The mercuric compound formed was collected on the filter and decomposed with hydrogen sulphide. The filtrate from the mercuric sulphide was evaporated to a syrup and dissolved in alcohol. To this solution alcoholic platinic chloride solution was added, and its mixture was allowed to stand. After forty-eight hours' standing, well known characteristic crystals of choline platinic chloride were formed. The crystals were collected on a filter, washed with alcohol, and dried over sulphuric acid in a vacuum. After drying, the weight of the substance was only 0.0613 gm., and its platinum content was found to be 31.27 per cent.

		Pt.
$(C_5H_{14}NOCl)_2PtCl_4$	Calculated:	31.66 per cent.
	Found:	31.27 per cent.

ON CREATINURIA.

By ALONZO ENGLEBERT TAYLOR.

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(Received for publication, May 4, 1915.)

The following data indicate that the presence of creatine in the urine of children cannot be regarded as related to deficiency of carbohydrate in the diet, as has been suggested; they also indicate that the finding of creatine in the urine cannot be ascribed to a methodic error, as has been suggested, due to the interference in the color reaction of the Folin method by acetone or acetoacetic acid. The subjects of this report were two little girls of ten and four years of age. They were not normal children, but this fact does not invalidate the conclusions to be drawn from the data. The elder child was the subject of hypopituitarism, the result of a tumor of unknown nature in the hypophysis. The younger child suffered from recurrent vomiting. The observations to be described were obtained in the period between attacks, when the child appeared entirely well. Both children were under strict observation of trained nurses, and there was every reason to believe that the instructions for feeding and collection of excrementa were carried out substantially as ordered. Both children were under metabolic tests, and the figures for creatine and creatinine are parts of the metabolic data.

The girl of ten weighed 110 lbs. The diet was a well balanced ration, containing on the average daily 59 gm. of protein, 58 gm. of fat, and 237 gm. of carbohydrate, the extreme figures for the latter being 210 and 259 gm. The creatinine was measured by the method of Folin, the creatine by the method of Benedict, the ketonic substances by the Shaffer method of triple distillation. The method of Benedict was found to work in a very satisfactory manner; though large amounts of urine had to be evaporated to dryness (25 cc.) a disturbing color never developed. The following figures cover the period of ten days' observation.

Days	1	2	3	4	5	6	7	8	9	10
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Creatinine	540	480	505	410	410	380	470	405	380	410
Creatine	40	40	135	110	95	90	110	105	140	110
Ketones*	210	240	205	150	170	180	175	165	—	175

* Ketones are in terms of β-oxybutyric acid.

The diet of the first child was not free of creatine-creatinine; it included each day 50 gm. of chicken breast or lamb chop; otherwise the diet consisted of cereals, vegetables, milk, eggs, bread, and butter.

The diet of the second child was almost free of fat, on account of the supposed influence of fat in the diet upon the formation of the ketonic acids. The child weighed 25 lbs. The diet consisted daily of about 30 gm. of protein, 200 gm. of carbohydrate and not over 10 gm. of fat, in the form of cereals, bread, milk, fruit, fresh vegetables, and a little meat once daily. The child was perfectly well upon this diet, but was not growing at the normal rate. The figures for the period of two weeks were as follows.

Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Creatinine	105	84	101	101	96	98	98	104	124	118	84	96	64	96
Creatine	95	78	39	69	56	50	42	56	42	36	48	54	68	108
Ketones	—	—	28	32	33	33	23	37	26	23	16	28	38	—

The figures for the urinary ketones in the first child are entirely normal; in the second child they are very low, due apparently to high carbohydrate and low fat intake. In each instance the intake of carbohydrate must be termed generous, even high, in relation to the total caloric intake. The constant creatinuria observed could not have been due to deficiency in carbohydrate intake. The amounts of ketones found in the urine were normal in the one child, very low in the second child, and could not have affected the colorimetric reading for creatinine, otherwise all normal urines would display a false creatinuria. Normal β-oxybutyric acid, as acid or salt, added to normal urine in the proportion of 1 gram to the liter has no effect upon the reading in the Folin method for creatinine. 1 gram of acetone or aceto-acetic acid, as salt, added to 1 liter of normal urine does affect slightly the colorimetric reading for creatinine, lowering the finding. One-fourth of a gram has no effect, one-half of a gram has no measurable effect. Ketones in the amounts stated could not therefore have affected the readings, particularly since about half

of the ketone was present as β-oxybutyric acid. Finally the highest relative findings for creatine were in the urine of the second child, which contained ketones in such mere traces that half the urine had to be distilled to make the estimations.

As control, four normal children were tested in the same manner, two girls of eight, two boys of five years. All were upon full normal diets.

	CREATINE	CREATININE	KETONES
	mg.	*mg.*	*mg.*
Girl	60	405	135
	65	555	225
Girl	100	380	235
Boy	35	295	65
	40	360	100
Boy	35	265	80

The mixed diets of these children contained notable amounts of meat, yet their urines contained no more creatine than was present in the urines of the two first children whose diets were low in meat. In particular, the first of the two girls last reported consumed a large amount of meat on the two days of the test, yet the figures for creatine were not high. The ketones averaged higher than in the urines of the two sick children.

Evidently there is a partition of creatine-creatinine in the urine of children, a partition naturally and reasonably to be ascribed to incomplete conversion of creatine into creatinine. Inanition and carbohydrate fasting in the adult will provoke creatinuria; but these data indicate that in the child creatinuria is not dependent upon low carbohydrate intake, persisting indeed in spite of high intake of carbohydrate. Of the total elimination in the elder child 19 per cent was in the state of creatine; in the younger child 40 per cent.

The absolute figures for creatine-creatinine compare approximately with those noted in adults, when the weights of the children and the probable masses of muscular tissue are considered, thus confirming for children the Folin-Shaffer postulate of the relation of creatinine to mass of muscle substance.

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